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(54) Title: GASTRIN-SPECIFIC INTERFERING RNA

(57) Abstract: The invention provides gastrin-specific interfering ribonucleic acid (RNAi) molecules that down-regulate the gastrin hormone gene. The RNAi molecules of the invention are double-stranded RNA molecules that can include modified bases and non-phosphate linkages between the bases. The complementary strands of the RNAi molecules of the invention can be linked by a nucleotide chain or by non-nucleotide linkers. The RNAi molecules of the invention can be formulated in pharmaceutical compositions useful in a method for treating gastrin-mediated diseases or conditions including gastrin-mediated tumors, GERD, Zollinger-Ellison syndrome, hypergastrinemia, pernicious anemia, gastric ulceration, duodenal ulceration, and *H. pylori* infection.

GASTRIN-SPECIFIC INTERFERING RNA

FIELD OF THE INVENTION

The present invention relates to short ribonucleic acid polymers that inhibit expression of the gene encoding the hormone, gastrin. In particular, the invention relates to inhibition of gastrin gene expression by short ribonucleic acid polymer molecules targeting gastrin messenger RNA (mRNA) to potentiate degradation and, in some cases, block translation so that expression is diminished or completely prevented.

BACKGROUND

Post-transcriptional gene-specific interference with the normal processes of expression by small ribonucleic acid molecules has been reported in many systems. Based on differences in structure and activity, these interfering RNA (RNAi) molecules may be divided into two major classes: small interfering RNAs and microRNAs.

Small interfering RNAs (siRNAs) are double-stranded RNA molecules generally consisting of paired ribonucleotide chains of about 22 nucleotides in length. Ruvkin, G. (2001) Glimpses of a Tiny RNA World. *Science* 294: 797-799. 27-mers have recently shown to be effective, for some targets. (Kim et al (2005) *Nature Biotechnology* 2:222). siRNAs were first identified in plants as molecules responsible for the phenomenon of co-suppression, now known to be a manifestation of post-transcriptional gene silencing. siRNAs have also been found in the fungus, *Neurospora*, shown to be responsible for the phenomenon of gene "quelling" and RNA interference in the fruit fly, *Drosophila*. For a review, see Calpen & Mousses (2003) *N.Y. Acad. Sci.* 1002: 56-62.

The presence of a novel RNase III-like enzyme having so-called "Dicer" activity was confirmed in each of these instances of gene silencing by siRNA. Dicer enzymatically cleaved double-stranded RNA yielding siRNAs with component RNA strands of less than 30 base pairs.

The siRNAs in turn were shown to act at a ribonucleic acid-protein complex called the RNA-induced silencing complex, or RISC, mediating specific targeting of mRNAs carrying the target sequence. Targeting is achieved by alignment of the mRNA with the antisense strand component of the siRNA in the RISC. Subsequently, the target mRNA is cleaved at a site within the target sequence, rendering it susceptible to rapid degradation by other cellular ribonucleases. See Denli and Hannon (2003) RNAi: An ever growing puzzle. *Trends Biochem. Sci.* 28: 196.

Vectors providing inducible expression of hairpin siRNAs have been constructed for knock-down of expression target genes. See for example Matsukura et al. (2003) Establishment of Conditional Vectors for Hairpin siRNA Knockdowns, *Nucl. Acids Res.* 31(15): e77.

MicroRNAs (miRNAs) are a second class of RNAi molecules distinct from siRNAs, hundreds of which have been identified in a diverse group of organisms from fruit flies, to worms and from plants to humans. See for example, Ambros, V. (2003) MicroRNA pathways in flies and worms. *Cell* 113: 673-676. MicroRNAs are 21-23 nucleotide single-stranded RNA molecules that are processed from larger double-stranded molecules, usually in the form of stem-loop structures. The double-stranded miRNA precursors are transcribed from non-protein-coding genes, forming self-complementary fold-back structures processed by "Dicer" enzyme.

MicroRNAs are critical for normal cellular differentiation and for development of multicellular structures. See Carrington and Ambros (2003) Role of microRNAs in Plant and Animal Development. *Science* 301:336-338. miRNAs are endogenous regulatory molecules that bind a target mRNA by hybridizing at an imperfectly matched sequence, preventing its translation. This gene-specific blockage of protein/peptide synthesis is in contrast to the degradation of the targeted mRNAs caused by siRNA molecules. This latter function appears to be dependent on a perfect or near-perfect matching hybridizing sequence of the anti-sense strand of the siRNA and the target sequence of the mRNA. MicroRNAs, by contrast are imperfectly matched to the target mRNA, hybridizing with the mRNA sequence in short stretches, without providing a sufficient length of double-stranded sequence for RNA degradation by RISC.

Efficacy of siRNAs targeted to different sites in the ICAM-1 mRNA has been demonstrated over a wide range of concentrations, and siRNAs have shown to be effective at nanomolar concentrations, a thousand-fold lower than the minimum concentration necessary for inhibition by antisense RNAs: Kretschmer-Kazemi & Sczakiel (2003) The Activity of siRNA in Mammalian Cells is Related to Structural Target Accessibility: A Comparison with Antisense Nucleotides, *Nucl. Acids Res.* 31(15): 4417-4424.

Long protein half-life and persistence of function even after complete ablation of *de novo* gene expression has been shown for the tumor-specific marker, NPM-ALK kinase protein. This protein is constitutively activated in anaplastic large cell lymphomas (ALCL), triggering malignant transformation. Ritter et al. (2003) Design and Evaluation of Chemically Synthesized siRNA Targeting the NPM-ALK Fusion Site in ALCL, *Oligonucleotides* 13: 365-373.

Small interfering RNA molecules have been shown to knock-down gene expression of the cancer related proteins, clusterin, IGFBP-2, IGBFBP-5, Mitf, and B-raf (See WO 2004/018676); growth factors such as VEGF, and other genes whose expression stimulates growth of tumors. See for example, Yin et al. SiRNA agents inhibit oncogene expression and attenuate human tumor cell growth (2003) *J. Exp. Therapeut. Oncol.* 3: 194-204. HeLa cells, lung adenocarcinoma cells, and melanoma cells were successfully transfected with siRNA-loaded cationic lipid complexes. However, even though these

growth factor gene specific siRNAs exhibited knock-down of their cognate targets, efficacy in suppression of proliferation of cancer cells differed. Cocktail combinations of five siRNAs were necessary to achieve significant inhibition of growth and proliferation of cancer cells by multigene knock-down.

Small interfering RNAs have been used in studies of a wide variety of cellular functions targeted for therapeutic intervention, including p53, TNF-alpha, *bcl-2*, and caspases in cell cycle regulation and apoptosis, growth factors and their regulation, protein kinases and signaling factors, RNA stability, DNA repair and tumor reversion. See for instance, Lu et al. (2003) siRNA-mediated anti-tumorigenesis for drug target validation and therapeutics, *Curr. Opin. Mol. Therapeutics* 5(3): 225-234; and Zender & Kubicka (2004) SiRNA based strategies for inhibition of apoptotic pathways *in vivo*-analytical and therapeutic implications, *Apoptosis* 9: 51-54.

Studies in model systems in which siRNA was delivered into specific organs *in vivo* have shown effective knock-down of expression of targeted endogenous genes or introduced genes. For example, expression of enhanced green fluorescent protein (eGFP) from a recombinant vector injected into the striatal region of mouse brain has been shown to be decreased by eGFP targeted siRNA. Similarly, an siRNA targeted to beta-glucuronidase in mouse liver was shown to cause a significant reduction in expression of the targeted gene. These are just two of many examples now available demonstrating *in vivo* efficacy of gene silencing by siRNA. See also, Xia et al. (2002) siRNA-mediated gene silencing *in vitro* and *in vivo*, *Nature Biotechnol.* 20: 1006-1010.

There is a need for novel treatments for gastrin-promoted diseases and conditions capable of reducing or even completely silencing gastrin gene expression in those patients whose conditions are exacerbated by greatly elevated levels of gastrin. Methods of reversibly regulating gastrin gene expression in these tissues would be a valuable adjunct to currently available therapies.

SUMMARY OF THE INVENTION

The present invention provides a gastrin gene-specific interfering ribonucleic acid (RNAi) molecule comprising a ribonucleotide chain of up to about 90 nucleotides in length including a targeting sequence of about 19 to 21 nucleotides, alternatively about 19 to 24, alternatively of about 19 to 27, alternatively of about 19 to 29 nucleotides of sufficient complementarity to a stretch of the coding sequence of the gastrin gene to bind gastrin mRNA (mRNA).

The invention also provides a double-stranded small interfering RNA (siRNA) molecule that inhibits expression of the gastrin hormone gene by RNA interference. The siRNA of the invention specifically interferes with gastrin gene expression and targets gastrin mRNA by a targeting sequence corresponding to the anti-sense strand sequence

complementary to a short stretch of from about 19 to about 21 nucleotides, alternatively about 19 to about 24 nucleotides, alternatively about 19 to about 27 nucleotides of the gastrin gene.

Also, the invention provides a gastrin gene-specific micro-ribonucleic acid (miRNA) molecule comprising a ribonucleotide chain of up to about 90 nucleotides in length including a targeting sequence of about 19 to about 21 nucleotides, alternatively about 19 to about 24 nucleotides, which can be up to about 27 nucleotides of that is partially complementary to a stretch of the coding sequence of the gastrin gene and binds gastrin mRNA (mRNA).

The invention further provides a pharmaceutical composition that includes a gastrin gene-specific interfering ribonucleic acid (RNAi) molecule including a ribonucleotide chain of up to about 90 nucleotides in length including a sequence of about 19-21 nucleotides, alternatively about 19-24 nucleotides, alternatively about 19-27 nucleotides, alternatively about 19-29 nucleotides of sufficient complementarity to a stretch of the coding sequence of the gastrin gene to bind gastrin mRNA (mRNA), and a physiologically acceptable carrier.

Further, the invention provides a method of treating a patient suffering from a gastrin-promoted disease or condition in a patient, wherein the method includes administering to the patient an effective amount of the gastrin gene-specific interfering ribonucleic acid (RNAi) molecule including a ribonucleotide chain of up to about 90 nucleotides in length including a sequence of about 19-21 nucleotides, alternatively about 19-24 nucleotides, alternatively about 19-27 nucleotides, alternatively about 19-29 nucleotides of sufficient complementarity to a stretch of the coding sequence of the gastrin gene to bind gastrin mRNA (mRNA).

Yet further, the present invention also provides a method of reducing gastrin gene expression in a cell, the method includes treating the cell with a gastrin-specific interfering ribonucleic acid (RNAi) molecule including a ribonucleotide chain of up to about 90 nucleotides in length including a sequence of about 19-21 nucleotides, alternatively about 19-24 nucleotides, alternatively about 19-27 nucleotides, alternatively about 19-29 nucleotides of sufficient complementarity to a stretch of the coding sequence of the gastrin gene to bind gastrin mRNA (mRNA).

Also provided is a nucleic acid vector expressing a gastrin-specific interfering ribonucleic acid (RNAi) molecule including a ribonucleotide chain of up to about 90 nucleotides in length including a sequence of about 19-21 nucleotides, alternatively about 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotides of sufficient complementarity to a stretch of the coding sequence of the gastrin gene to bind gastrin mRNA (mRNA).

Further, the invention provides a host cell carrying a nucleic acid vector expressing a gastrin-specific interfering ribonucleic acid (RNAi) molecule including a ribonucleotide chain of up

to about 90 nucleotides in length including a sequence of about 19-21 nucleotides, alternatively about 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotides of sufficient complementarity to a stretch of the coding sequence of the gastrin gene to bind gastrin mRNA (mRNA).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at day 1 following transfection of Pan1 cells with tg5 or scrtg5 siRNA using 1, 2 or 4 μ l transfection reagent per transfection.

Figure 2 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at day 1 following transfection of HCT116 cells with tg8 or scrtg8 siRNA using 1, 2 or 4 μ l transfection reagent per transfection.

Figure 3 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at day 1 following transfection of Pan1 cells with the full range of *in vitro*-synthesised gastrin siRNAs at a range of concentrations: undiluted (20nM), 1:3 (6nM) and 1:10 (2nM).

Figure 4 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at day 1 and day 4 following transfection of Pan1 cells with the full range of *in vitro*-synthesized gastrin siRNAs (20nM).

Figure 5 Gastrin gene expression at day 1 following transfection of Pan1, HCT116, MGLVA1 or OE19 cells with commercially-synthesized tg8 or scrtg8 siRNA at 40nM (undiluted) or 4nM (1:10 dilution). Data based on: a) the $2^{-\Delta\Delta Ct}$, and b) the $2^{-\Delta Ct}$.

Figure 6 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at day 1 following transfection of HCT116 cells with serial 1:4 dilutions of commercially-synthesized tg8 or scrtg8 siRNAs starting at a concentration of 40nM.

Figure 7 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at days 1, 4, 7 and 11 following transfection of Pan1 cells with commercially-synthesized tg8 or scrtg8 siRNAs at a concentration of 40nM (undiluted) or 4nM (1:10 dilution).

Figure 8 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at days 1, 3, 6 and 9 following transfection of HCT116 cells with commercially-synthesized tg8 or scrtg8 siRNAs at a concentration of 40nM (undiluted) or 4nM (1:10 dilution).

Figure 9 Down-regulation of gastrin gene expression by gastrin siRNA in the presence of EGF. Gene expression in PAN-1 cells on day 2 after transfection with gastrin (tg8) or control (scrtg8) siRNA and 24hrs treatment with or without 10 μ g/ml EGF. Data are expressed relative to the housekeeping gene, HPRT. Error bars indicate the 95% confidence intervals. Significant differences ($p<0.001$) are indicated by *.

Figure 10 Inhibition of GFP-tagged gastrin protein expression by gastrin siRNA. HCT116 cells were transfected with a plasmid expressing GFP-tagged gastrin together with the control scrtg8 siRNA (a) or gastrin tg8 siRNA (b). The percentage of positive cells is indicated. There was a significant difference between the two treatment ($p<0.0001$)

Figure 11 Inhibition of growth by gastrin siRNA. Growth of PAN-1, C170HM2, HCT116, and MGLVA1 cells in serum-free medium (a) with or (b) without the addition of 10 μ g/ml

EGF following transfection with gastrin (tg8) or control (scrtg8) siRNAs. Growth of tg8-transfected cells is shown as a percentage of scrtg8-treated cells. Significant differences ($p<0.05$) are indicated by *.

Figure 12 Apoptosis in gastrin siRNA-transfected cancer cells

Proportion of apoptotic cells at d4 following treatment with gastrin (tg8) or the control (scrtg8) siRNA (a) in the presence or (b) absence of 10 μ g/mlEGF. Apoptosis is shown relative to scrtg8-treated cells. Significant differences ($p<0.05$) are indicated by *.

Figure 13 Interference with the OE19 autocrine gastrin pathway using anti-gastrin (Tg8) siRNA. A Western blots showing levels of total PKB/Akt and phospho-PKB/Akt in OE19 cells following treatment with anti-gastrin siRNA. Duplicate transfections were carried out for each treatment condition; Lanes 1 & 2 = OE19 + 500ng/ μ l (1x) Tg8, 3 & 4 = OE19 + 50ng/ μ l (1/10x) Tg8, 5 & 6 = 500ng/ μ l (1x) SNC, 7 & 8 = OE19 50ng/ μ l (1/10x) SNC.

B Summary graph showing ratio of phospho-PKB/Akt to total PKB/Akt in the various treatment groups as determined via densitometry. (500ng/ μ l Tg8 compared to 500ng/ μ l SNC $p=0.003$)

Figure 14 HB-EGF gene expression in ST16, AGS or MGLVA1 cells treated with the gastrin siRNA (target 8) or control siRNA (scrambled target 8)

Figure 15 HB-EGF gene expression in ST16, AGS or MGLVA1 cells treated with the control siRNA alone (scrambled target 8); the control siRNA (scrambled target) and H.pylori strain 60190; or gastrin siRNA (target 8) and H.pylori strain 60190.

Figure 16 HB-EGF ectodomain shedding in response to H. pylori strain 60190 in the presence or absence of the gastrin siRNA (target 8) or the control siRNA (scrambled target 8)

Figure 17 XIAP gene expression in AGS cells treated with the gastrin siRNA (target 8) or the control siRNA (scrambled target 8)

Figure 18 XIAP gene expression in AGS cells treated with the gastrin siRNA (target 8) or the control siRNA (scrambled target 8) and then exposed to H. pylori strain 60190 for 24hrs

Figure 19 XIAP protein expression in AGS cells treated with H. pylori strain 60190 in the presence or absence of the gastrin siRNA (target 8) or the control siRNA (scrambled target 8)

Figure 20 Gastrin gene expression ($2^{-\Delta\Delta Ct}$) at days 1 following transfection of C170HM2 cells with commercially-synthesized Gas-27 or scrtg8 siRNAs at a range of concentrations between 40 and 0.1nM.

Figure 21 Vector (pSilencer 2.1-U6 hygro, Ambion) used to express gastrin and control siRNAs as small hairpin RNAs. Insert structure and resulting small hairpin RNA are also illustrated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an interfering RNA (RNAi) molecule that includes a ribonucleotide chain of no more than about 90 nucleotides, wherein a stretch of about 19 to 21 nucleotides, alternatively 19 to about 24 nucleotides, alternatively about 19 to 27 nucleotides, alternatively about 19-29 nucleotides is sufficiently complementary to a sequence within the sense-strand of the gastrin gene such that the sequence of about 19-21 nucleotides, alternatively about 19-24 nucleotides, alternatively about 19-27 nucleotides, alternatively about 19-29 nucleotides binds gastrin mRNA. This stretch of about 19 to 21 nucleotides, alternatively about 19 to about 24 nucleotides, alternatively about 19-27 nucleotides, alternatively about 19-29 nucleotides is a gastrin gene targeting sequence that directs the RNAi molecule to bind gastrin mRNA. The gastrin gene targeting sequence may be complementary to any sequence of about about 19 to 21 nucleotides, alternatively about 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotides within the sequence transcribed from the gastrin gene. Preferably, the gastrin gene targeting sequence is between about 20 to about 21 nucleotides long. More preferably, the gastrin gene targeting sequence is 21 or 22 nucleotides long. More preferably, the gastrin gene targeting sequence is up to 29 nucleotides long.

The gastrin gene-specific interfering RNA targeting sequences of the invention preferably have no more than about 80 percent complementarity with any other gene, especially any other human gene, i.e. when aligned base for base, considering for example, a gastrin targeting sequence of 20 nucleotides in length, no more than about 16 of nucleotides are complementary to any other gene, especially any other human gene. More preferably, no more than 70 percent of the nucleotides of the gastrin gene-specific interfering RNA targeting sequences are complementary to any other gene, especially any other human gene.

The targeting sequence preferably has a GC content of from about 30 and about 60 percent, has no more than about four consecutive adenosine bases.

The gastrin-specific interfering RNA (RNAi) molecules of the present invention comprises a chain of ribonucleotides, i.e. a 3'-5' phosphodiester linked pentose sugar-phosphate backbone, preferably, each sugar being substituted at the 1'-position with a nucleotide base. The ribonucleotide chain may include one or more linkages which are not phosphate diester linkages, such as for instance linkages containing a sulfur atom, a nitrogen atom or a carbon atom to prevent *in vivo* nuclease cleavage and degradation. Preferably the nucleotide base is adenine, guanine, cytidine or uracil. The sugar may be any pentose sugar, but is preferably D-ribose. The ribonucleotide chain of the RNAi molecules of the present invention can include one or more modified nucleotide bases, preferably a modified purine base or a modified pyrimidine base. The modified purine base may be any modified purine base, for example a hypoxanthine (inosine in the ribonucleotide chain), or a modified purine

base, for instance a methylated adenine, a substituted guanine base, or the like. The modified pyrimidine base may be any modified pyrimidine base, such as a modified cytidine or uracil, such as 5-methyl cytosine or the like.

In one embodiment, the RNAi molecule of the invention includes a gastrin gene targeting sequence no more than about 19- 21 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotides that is perfectly complementary to a sequence within the sense-strand of the gastrin gene. When bound to gastrin mRNA, every one of the ribonucleotide bases of the gastrin gene targeting sequence of this embodiment of the gastrin-specific RNAi is paired in a GC or a CG pair, or in an AU or an UA base pair with the complementary base in the gastrin mRNA.

In another embodiment of the RNAi molecule of the present invention, one or more mismatches in the approximately 19-21 nucleotides, alternatively 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotide gastrin gene targeting sequence are tolerated provided that the sequence within the gastrin gene targeting sequence that is perfectly complementary to the sense-strand of the gastrin gene is sufficient to form a stable double stranded RNA with gastrin mRNA. Estimation of the limits of such complementarity required for stable double strand formation (i.e. melting temperature profile) is routine and well within the ordinary skill in the art. See for example, Sambrook & Russell (2000) Molecular Cloning, A Laboratory Manual. 3d edn. Cold Spring Harbor Laboratory Press, Chapter 10: Working with Synthetic Oligonucleotide Probes. Thus, for example, the gastrin gene targeting sequence of the RNAi of the invention may include one, two, three or four mismatched bases may be included in the gastrin gene targeting sequence, leaving about 18 to about 21, about 17 to about 24, or about 15 to about 27 nucleotides, or about 16 to about 29 nucleotides perfectly matched with the sense-strand sequence.

The RNAi molecule of the present invention may include no more than four bases in the approximately 19-21 nucleotides, alternatively 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotide gastrin gene targeting sequence that are mismatched with the targeted sequence from within gastrin gene. The maximum permissible number of mismatches between the gastrin gene-specific targeting sequence of the RNAi of the invention and the gastrin gene that still allows hybridization can be determined by routine methods in the known to those of skill in the art. See for example Urakawa et al. (2003) Optimization of Single-Base-Pair Mismatch Discrimination in Oligonucleotide Microarrays. *J. Appl. Envir. Microbiol.* 69(5): 2848-2856.

In one embodiment, the ribonucleotide chain of no more than about 90 nucleotides of the gastrin gene-specific RNAi molecule of the invention includes a second nucleotide sequence of between about 19 and about 21 nucleotides, alternatively about 19 and about 24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotides having sufficient complementarity to bind the approximately 19 to approximately 21 nucleotides,

alternatively 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotide gastrin gene targeting sequence. Preferably, this complementarity is complete sequence complementarity such that every base of the approximately 19 to approximately 21 nucleotides, alternatively 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotide gastrin gene targeting sequence is paired with the second nucleotide sequence in a GC or an AU base pair. Alternatively, the second nucleotide sequence may include one, two, three or more bases mismatched with the opposing base in the gastrin gene targeting sequence in the double stranded gastrin gene-specific interfering ribonucleic acid molecule. Preferably, the second nucleotide sequence includes no more than about 9 mismatched bases in the gastrin gene targeting sequence. More preferably, no more than about 6 mismatched bases and most preferably, no more than about 3 mismatched bases are included in the gastrin gene targeting sequence.

Individual strands of double-stranded embodiments of the gastrin-specific interfering RNA of the invention, whether siRNA or miRNA, may be chemically synthesized *in vitro* or expressed from a recombinant clone and annealed to form the double-stranded RNAi molecule. Alternatively, the gastrin-specific interfering RNA of the invention may be synthesized or expressed as a single strand with the complementary sequences connected by a loop sequence. The complementary sequences can then be folded back and annealed under appropriate conditions to form a single molecular siRNA species known as a double-stranded "hairpin siRNA" molecule. The two complementary strands of the small interfering RNA can be linked by an oligonucleotide linker, or by a non-nucleotide linker. Suitable non-nucleotide linkers, for instance short inorganic polymer linkers, peptide linkers, such as polyglycine or polylysine linkers and the like are well known in the art and are readily adapted to *in vitro* RNAi synthesis by chemical and or enzymatic methods.

In a particular embodiment, the gastrin-specific interfering RNA of the invention comprises two separate ribonucleotide chains forming a double-stranded RNAi molecule. One ribonucleotide chain that includes the gastrin anti-sense targeting sequence of approximately 19-21 nucleotides, alternatively 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotides in length and a second ribonucleotide chain having a sequence complementary or at least partially complementary to the gastrin anti-sense targeting sequence. In the siRNA embodiment, the ribonucleotide chain that includes the gastrin anti-sense targeting sequence and the second ribonucleotide chain are fully complementary along the full length of the approximately 19-21 nucleotides, alternatively 19-24 nucleotides, alternatively 19-27 nucleotides, alternatively 19-29 nucleotide gastrin gene-specific targeting sequence. The double-stranded RNAi molecule can also include an unpaired "overhang" ribonucleotide sequences at one or more of the four ribonucleotide chain ends. The overhang at any particular end of the ribonucleotide chain can be of any

length such that the total number of ribonucleotides in the molecule does not exceed about 90 in all.

In a preferred embodiment, the gastrin-specific double-stranded RNAi molecule of the invention includes an overhang sequence of ribonucleotides at the 3' end of one or both of the ribonucleotide chains or alternatively comprises a blunt-end sequence. In a particularly preferred embodiment, the gastrin-specific double-stranded RNAi molecule of the invention includes a overhang of one, two or three ribonucleotides at the 3' end of one or both of the ribonucleotide chains. In an especially preferred embodiment, the gastrin-specific double-stranded RNAi molecule of the invention includes an overhang of two bases at the 3' end of both of the ribonucleotide chains and no overhang at the 5' ends of the chains. Embodiments of the double-stranded RNAi molecule of the invention that include unpaired ribonucleotide overhangs at all four ends of the two chains are also contemplated within the scope of the present invention. Such unpaired ribonucleotide overhangs form "ragged ends" of the RNAi molecule which may be cleaved by ribonucleases *in vivo* to form the active RNAi species.

The gastrin-specific RNAi of the invention can be expressed as a single stranded ribonucleotide structure or as a hairpin ribonucleotide structure from an inducible recombinant gene. The recombinant gene can be expressed from a DNA vector such as a DNA plasmid, or a DNA virus vector, or alternatively, the recombinant gene can be expressed from an RNA viral vector such as a retrovirus vector or the like. The knock-down of target gene expression can then be achieved by the single step of inducing the expression of the recombinant RNAi gene. This targeted-knock-down can be reversed by shutting down the expression of the RNAi and thereby permitting the gastrin gene to be expressed normally.

RNA interference with gene expression is manifested at the level of protein synthesis as a reduction in the expression of the protein or peptide product of the target gene. This down-regulation of gene expression of the target gene by an RNAi molecule exhibits stringent sequence specificity and is referred to as "knock-down" of the expression of the target gene, in contrast to the "down-regulation" mediated by anti-sense RNA molecules and the "knock-out" seen after deletion of the target gene. The knock-down of gastrin gene expression may be partial inhibition of expression of gastrin gene products, or may be complete ablation of expression of gastrin gene products, referred to as "silencing" of the gastrin gene.

Gastrin-specific gene products include all gastrin-specific peptide translation products, for instance, gastrin mRNA transcripts and the peptide products of translation of gastrin mRNA. The peptide products of gastrin mRNA translation include for instance, preprogastin (the primary translation product including a leader sequence for transmembrane transport of the peptide); progastin (the gastrin precursor pro-peptide which is produced from preprogastin by cleavage of the leader sequence and is matured to one or

more of the gastrin hormone forms by specific proteolytic cleavage); the gastrin hormone forms: gastrin 17, and gastrin 34 named according to the number of amino acids in the peptide chains; and glycine-extended gastrin 17 and glycine-extended gastrin 34, having a glycine residue extension to the C-terminal of gastrin 17 or gastrin 34, respectively; modified forms such as sulfated forms of each of the above gastrin hormone forms; and the C-terminal flanking peptide. These gastrin hormone forms exhibit differing biological activities. For instance, G17-Gly and G34-Gly are implicated in stimulation of growth and proliferation of gastrin-promoted neoplasms through activating a receptor that specifically binds the glycine-extended C-terminal sequences of these gastrin hormone forms.

Complete knock-down of gastrin gene expression can be achieved by administering an effective amount of a gastrin-specific interfering RNA of the invention selected for the ability to cause complete blockage of gastrin hormone synthesis, and which may be accompanied by targeted degradation of gastrin mRNA as explained below. The siRNAs of the present invention are useful as therapeutic agents for inhibition of gastrin-promoted tumor growth and proliferation. Complete knock-down of gastrin gene expression may be necessary for gastrin-promoted tumors and other conditions stimulated by an autocrine mechanism, where the diseased tissue itself produces gastrin that promotes its own growth and proliferation. Alternatively, complete knock-down of gastrin gene expression by the gastrin-specific RNAi may be targeted to a different tissue that produces gastrin in a paracrine mechanism.

Alternatively, partial knock-down of the gastrin mRNA can be achieved by administering an effective amount of a gastrin-specific interfering RNA of the invention that leaves residual gastrin gene expression, due to administration of a dose of RNAi of the invention that results in under titration of the gastrin mRNA, or is targeted to a sequence that is ineffective in completely blocking gastrin gene expression. Partial knock-down of the gastrin gene expression may be advantageous in the treatment of gastrin-promoted diseases and conditions such as Zollinger-Ellison syndrome due to overproduction of gastrin. Reduction in the level of gastrin production to normal levels in such diseases and conditions can be achieved by knock-down of gastrin expression by administration of an effective amount of the gastrin-specific interfering RNA according to the methods of the present invention and as exemplified below.

An effective amount of a gastrin-specific RNAi molecule according to the present invention is that amount which when administered to a patient suffering from a gastrin-promoted disease or condition causes a knock-down of gastrin gene expression. The knock-down of gastrin gene expression may occur only at the translational level, i.e. blocking or reducing synthesis of gastrin peptide products. Alternatively, the knock-down of gastrin gene expression may include targeted degradation of gastrin mRNA, as well as blocking or reducing its translation. In one embodiment, the knock-down of gastrin gene expression

may be a complete knock-down in which all, or substantially all gastrin gene expression is prevented.

In another embodiment, the knock-down of gastrin gene expression caused by administration of a gastrin-specific RNAi molecule according to the present invention can be a partial knock-down wherein gastrin gene expression is reduced but not completely prevented. For instance, the partial knock-down of gastrin gene expression can be a reduction of about 25 percent in the level of gastrin peptide products of the gastrin gene. Preferably, the partial knock-down of gastrin gene expression is a reduction of about 50 percent in the level of gastrin peptide products of the gastrin gene. More preferably, the partial knock-down of gastrin gene expression is a reduction of about 75 percent in the level of gastrin peptide products of the gastrin gene. Yet more, preferably, the partial knock-down of gastrin gene expression is a reduction of about 85 percent in the level of gastrin peptide products of the gastrin gene.

Additionally, the level of gastrin mRNA may be reduced by administration of a gastrin-specific RNAi molecule of the present invention. For instance, the level of gastrin mRNA can be reduced by about 25 percent, preferably by about 50 percent, more preferably by about 75 percent, and most preferably by about 85 percent upon administration of the gastrin-specific interfering RNA of the present invention. Optimally, administration of a gastrin-specific interfering RNA of the present invention causes complete, or substantially complete (i.e. about 90-100 percent) degradation of gastrin mRNA.

Combinations of gastrin-specific interfering RNAi molecules targeted to different gastrin sense-strand sequences can be administered to a patient suffering from a gastrin-promoted disease or condition in order to improve therapeutic efficacy by augmenting the knock-down of gastrin gene expression achieved with each of the RNAi molecules alone.

In another embodiment, the invention provides a method of using a gastrin-specific RNAi molecule in combination with a chemotherapeutic agent to knock-down expression of gastrin for the treatment of gastrin-promoted gastrointestinal tumors in concert with chemotherapy. The chemotherapeutic agents suitable for use in combination with the gastrin-specific RNAi of the present invention may be any chemotherapeutic agent, such as for instance gemcitabine, camptothecin, doxorubicin, 5-fluorouracil (5FU), docetaxel, paclitaxel, vinblastine, etoposide (VP-16), oxaloplatin, carboplatin, cisplatin (CDDP), a kinase inhibitor (e.g. erlotinib, Iressa®), an angiogenesis inhibitor (e.g. Bevacizumab), and an EGF receptor inhibitor (e.g. cetuximab).

The chemotherapeutic agent may be administered at any stage of a gastrin-specific RNAi therapy regimen according to the methods of the present invention, including continuously or intermittently during the gastrin-specific RNAi therapy regimen.

Methods treatment of gastrin-promoted tumors of the present invention include methods wherein a gastrin-specific RNAi molecule is administered directly at the site of the

tumor. Alternatively, the gastrin-specific RNAi molecule can be administered at a distant site. The treatment can be administered in conjunction with a chemotherapy regimen.

Gastrin-promoted diseases or conditions amenable to treatment with a gastrin-specific RNAi of the present invention include all gastrin-promoted tumors (both GI and non-GI), gastric-esophageal reflux disease (GERD), premalignant conditions like for example Barrett's esophagus, atrophic gastritis, gastric ulceration, duodenal ulceration, hypergastrinemia, such as in Zollinger-Ellison syndrome, pernicious anemia and *Helicobacter pylori* infection. Gastrin-promoted tumors treatable with a gastrin-specific RNAi of the present invention include colonic adenomas, pan-intraepithelial neoplasias, esophageal tumors, gastric neoplasias, intestinal tumors, pancreatic tumors, small cell lung cancers, medullary thyroid carcinomas, hepatic tumors, pulmonary tumors, ovarian tumors, glioblastomas, astrocytomas, tumors of brain origin such as glioblastomas or astrocytomas and tumors of neuroendocrine origin.

The gastrin-specific RNAi molecule according to the present invention is preferably administered to a patient in a pharmaceutical composition. The gastrin-specific RNAi molecule can be administered as a bolus or continuously, either systemically or locally at the site of the tissue that responds to gastrin. As used herein, a patient is an animal or a human suffering from a gastrin-promoted disease or condition. The disease can also be any disease where the gastrin gene is upregulated by growth factors, environmental stimuli or transcription factors.

The pharmaceutical compositions useful in the practice of the methods of the present invention include a gastrin-specific RNAi molecule and a physiologically compatible carrier or excipient. Suitable carriers or excipients include any physiologically compatible buffer, such as, for instance phosphate buffered saline (PBS).

The pharmaceutical compositions useful can further have a peptide covalently linked to the RNAi molecule. For example, these could be peptides such as TAT which enable uptake into cells (Cell. 1988 Dec 23;55(6):1189-93). These peptides are used for increasing transport into cells e.g. liposomes (PNAS 2001 98:8786-8781). Exemplary, penetratin and transportan have also been tried for siRNAs (Muratovska 2004 FEBS Letters 558:63-68). Alternatively, peptides could be used that would allow targeting to particular cell-types e.g. gastrin peptide to get the siRNAs into gastrin receptor-expressing cells. The peptides could be either directly linked to the siRNA or attached to the carrier (polymer/liposome etc).

In another embodiment, the pharmaceutical compositions useful in the practice of the methods of the present invention include a nucleic acid vector expressing a gastrin-specific RNAi molecule according to the present invention and a physiologically compatible carrier or excipient.

In yet another embodiment, the pharmaceutical compositions of the present invention include a recombinant vector expressing a gastrin-specific RNAi of the present invention as a single stranded ribonucleotide structure or as a hairpin ribonucleotide structure from an inducible recombinant gene, and a physiologically compatible carrier or excipient.

Incorporation by reference: The texts of each of the patents and other publications cited in this specification are hereby specifically incorporated by reference in their entireties.

EXAMPLES

EXAMPLE 1. siRNA synthesis

An *in vitro* system for siRNA synthesis (marketed by Ambion Ltd., Huntingdon, Cambridgeshire, UK) was used, in which the two strands of the siRNA are synthesized using T7 polymerase, annealed together to form a double stranded structure and purified by glass fiber filter binding and elution. The concentration of the double stranded nucleotide was calculated by measurement of the absorbance at 260nm.

A fluorescent siRNA was synthesised by modification of the above method, involving addition of fluorescein-12-uridine-5'-triphosphate during single strand synthesis. This required optimization of the ratio of fluorescent to non-fluorescent nucleotides and use of an increased overall concentration of ribonucleotide triphosphates (rNTPs). The higher concentration of rNTPs gave a higher efficiency of siRNA synthesis which may also be applicable to synthesis of non-fluorescent siRNAs.

Alternatively, siRNAs were synthesized commercially (Dharmacon, Lafayette, CO, USA). The two strands were synthesized separately using standard RNA oligonucleotide chemistry, converted to the 2'-hydroxyl form and annealed. The duplex was PAGE – purified and desalted by standard methods well known in the art.

Example 2: Cell-lines

The following cell-lines were used:

- a) Pan1: - a human pancreatic adenocarcinoma cell-line.
- b) HCT116 – a poorly differentiated human colon adenocarcinoma cell-line (ECACC, ref.no. 91091005)
- c) MGLVA1 – a gastric adenocarcinoma cell-line, an ascitic variant of the gastric cell line MKN45G (Watson et al., 1990. Int.J.Cancer, 45, 90-4)
- d) OE19 – an esophageal adenocarcinoma cell line.
- e) C170HM2 – human colorectal adenocarcinoma cell line originally derived from a poorly differentiated tumor with the ability to metastasise to the liver (Watson et al., 1993, Eur.J.Cancer, 29A,1740-5)
- f) AGS - human gastric adenocarcinoma cell line (ECACC, Wiltshire UK)

g) ST16 - human gastric adenocarcinoma cell line (Academic Unit of Cancer Studies, University of Nottingham)

All cell lines were routinely cultured in RPMI 1640 culture medium (Gibco, Paisley, UK) containing 10% (v/v) heat inactivated foetal bovine serum (FBS, Sigma, Poole, UK) at 37°C in 5% CO₂ and humidified conditions.

Example 3: siRNA transfection

Transfection reagents were from Ambion (Ambion Ltd., Huntingdon, Cambridgeshire, UK). In experiments with Pan1 cells, siPort Amine (Ambion Ltd.) gave good transfection efficiencies with relatively low cell toxicity, whilst siPort Lipid (Ambion Ltd.) was apparently extremely toxic to the cells.

The fluorescent siRNA was used to check the efficiency of transfection. Almost 100% of the cells were fluorescent when viewed through a fluorescent microscope 24hrs after transfection. The distribution of fluorescence in the cells transfected with fluorescently labeled siRNA, tg5, was examined using confocal microscopy. Fluorescence was associated with vesicle-like structures in the cytoplasm.

Example 4: Gastrin gene expression

A series of gastrin siRNA transfections of cell-lines was undertaken. For several of these experiments gastrin gene expression was measured using real-time PCR. Gastrin gene expression in cells treated with gastrin siRNAs was calculated relative to cells treated with the scrambled tg8 siRNA (scrtg8) and the housekeeping gene, hypoxanthine-guanine phosphoribosyl transferase (HPRT) or relative to the housekeeping gene alone using the "2^{-ΔΔCt} method" and the "2^{-ΔCt} method" respectively.

The so called "2^{-ΔΔCt} method" was generally used to calculate and standardize the amount of target in a sample. See Perkin Elmer Applied BioSystems User Bulletin #2 "ABI Prism 770 Sequence Detection System: Relative Quantitation of Gene Expression." Issue of December 1, 1997. This publication describes methods for monitoring and standardizing gene expression after transfection with siRNA using the expression level of any of a number of housekeeping genes e.g. beta-actin, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal RNA (rRNA), or other RNAs as endogenous baseline control.

The term Ct is the "threshold cycle" where the relative copy number is seen to breakthrough into a detectable exponential phase of amplification.

According to the 2^{-ΔΔCt} method, the amount of target for a sample (q), normalized to the endogenous reference and relative to a calibrator (cb) is given by

2^{-ΔΔCt}, where

$$\Delta\Delta C_t = \Delta C_{t,q} - \Delta C_{t,cb}$$

ΔC_{t,q} is the difference in threshold cycles for targets T and q;

and

$\Delta C_{t,cb}$ is the difference in threshold cycles for targets t and calibrator, cb.

This latter method compensates for differences between the reference control sample and the test samples for factors such as precise cell numbers, and efficiency of RNA and cDNA synthesis ($\Delta C_{t,cb}$). The method also provides a measure of gene expression in the test sample relative to the control-treated sample ($\Delta C_{t,cb}$).

In the $2^{-\Delta C_t}$ method, $\Delta C_t = C_{t,X} - C_{t,cb}$ (i.e. the difference in threshold cycles for the target and the housekeeping genes). This method is used to illustrate differences in absolute expression levels, for example between different cell lines.

Example 5: Titration of transfection reagent

Different quantities of the siPort Amine (Ambion, UK) transfection reagent were used to transfect Pan1 cells, referred to in the figures as A1, A2 and A4, corresponding to 1 μ l, 2 μ l and 4 μ l of the siRNA reagent per transfection. Gastrin gene expression monitored by PCR using the $2^{-\Delta C_t}$ method is shown in Figure 1.

The experiment was repeated using commercial siRNAs and HCT 116 cells. Results are shown in Figure 2. Thus, the highest knock-down of gastrin gene expression is generally seen with the highest quantity of transfected siRNA (by siPort Amine), i.e. 4 μ l per transfection in these experiments.

Example 6: Choice of targets within the gastrin gene

Eight siRNAs were tested, including six different targets within gastrin and two control siRNAs targeting scrambled (scr) versions of two of the gastrin siRNAs. The targets were chosen for the following properties:

- a) preceded by the dinucleotide AA in the gene sequence;
- b) avoiding the extreme 5' or 3' ends of the gene;
- c) low GC content (approximately 30-60%)
- d) absence of long stretches of A's; and
- e) lack of significant homology with other genes.

The sequences, GC content and position within the gene of each siRNA are given below in Table 1. None of the sequences showed significant homology with any other known genes as determined by BLAST analysis.

Table 1 Gastrin siRNA target sequences

Target	Target Sequence	%GC	Position in gene	SEQ ID NO.
tg1	AAGCTTCTTGGAGGCCCGCT	57.1	59	1
tg5	AAGAAGCAGGGACCATGGCTG	57.1	220	2

tg7	AAGAAGAAGAAGCCTATGGAT	38.1	245	3
tg8	AAGAAGAAGCCTATGGATGGA	42.9	248	4
tg9	AAGAAGCCTATGGATGGATGG	47.6	251	5
tg10	AAGCCTATGGATGGATGGACT	47.6	254	6
scr tg5	AAGAGATGTAAGGCCAGGCCG	57.1	n/a	7
scr tg8	AAGCGAAGAACGAGGTGTAT	42.9	n/a	8
Gas- 27	AAGAAGAAGCCTATGGATGGATGGA CT	44.4	248	9

Targets tg5 and tg7 were initially investigated as the best potential targets since target 7 had the lowest GC content of the targets in the 3' end of the gene whilst target 5 targeted an independent region of the gene compared with target 7, but avoided the extreme 5' end of the gene. Approximately 85% knock-down of gene expression was achieved with tg5 whilst tg7 gave more variable and generally lower knock-down. The remaining targets were then assessed for knock-down of gastrin gene expression. Target tg1 is located towards the 5' end of the gene, which has generally been found to be less successful in other siRNA gene silencing systems. Targets 8, 9 and 10 are immediately adjacent to tg7 with only a three nucleotide shift between each target sequence.

The gastrin siRNAs tg1, tg5, tg7, tg8, tg9 and tg10, and the scrtg5 control siRNA were synthesized using the *in vitro* method described above and tested on Pan-1 cells at a range of concentrations (undiluted, 1:3 and 1:10 dilutions, corresponding to final concentrations of approximately 20nM, 6nM and 2nM respectively). Transfections were carried out using siPort Amine (Ambion). RNA was extracted using RNABee (Ambion), cDNA synthesised using Superscript II (Invitrogen, Carlsbad, California) and random hexamers as primers. Gastrin and hypoxanthine-guanine phosphotransferase (HPRT) gene expression was measured in each set of cells by real-time PCR. HPRT, a cellular housekeeping gene function was chosen as a control gene that should not be specifically affected by RNA interference targeted to the gastrin gene. The results are shown in Figure 3, expressed relative to the scrambled siRNA control, using the $2^{-\Delta\Delta C_t}$ method, with error bars showing the 95% confidence intervals about the means.

The most effective targets were tg8 and tg9, providing 95% knock-down of gastrin gene expression. The siRNA, tg1 gave the least knock-down of gastrin gene expression. Knock-down by the scrambled tg5 siRNA was lower (about 30%). For each of the siRNAs, the knock-down effect decreased with decreasing concentrations of the transfected siRNA.

The above transfections were repeated in duplicate, allowing harvesting of the cells on day 1 and day 4 following transfection. The results are shown in Figure 4. The ranking of the effectiveness of the different siRNAs was the same in this experiment on day 1 as observed in the previous experiment (described above). Gastrin gene expression was

knocked down and stayed low over time with siRNAs tg8 and tg9, but substantial recovery of gastrin gene expression was observed with siRNAs, tg7 and tg10. These data confirm tg8 as the most effective anti-gastrin siRNA with tg9 as the next most effective alternative.

In order to investigate reasons for the differences in activity of the different targets, the RNA structure of gastrin mRNA was predicted using the RNA2 software available at (http://www.genebee.msu.su/services/rna2_reduced.html).

The siRNAs, tg7, tg8, tg9, and tg10 appear to target the same loop structure within the gastrin mRNA. However, tg5 targets a different structure in the precursor RNA from that targeted by tg7, tg8, tg9, and tg10. Nevertheless, hybridization of the antisense strands of tg7, tg8, tg9, or tg10 would involve opening up similar structures within the RNA to allow targeting by the RISC, believed to be the complex responsible for mediation of gene silencing.

Example 7: Effect of gastrin siRNAs on a range of cell-lines

All of the experiments described up to this point were carried out on Pan-1 cells, a pancreatic tumor cell-line. An experiment was set up to test the commercial siRNAs on four additional cell lines: HCT116 (colon), MGLVA1 (gastric), OE19 (oesophageal) and C170HM2 (liver metastasis of colonic).. The PCR data monitoring gastrin mRNA as described above, are shown below. In Figure 5a, the data is shown using the $2^{-\Delta\Delta Ct}$ calculation method taking into account the effects due to control siRNA and the effects on the control HPRT gene; and in Figure 5b the absolute gastrin levels are shown using the $2^{-\Delta Ct}$ method.

In the HCT116, MGLVA1 and C170HM2 cell-lines, as with the Pan1 cells, approximately 95% knock-down of the gastrin gene expression was achieved. Sixty percent knock-down was achieved with the OE19 cells. Figure 5b shows that the absolute amount of gastrin mRNA expressed by OE19 cells is considerably higher (approximately 150-fold higher) than that of the Pan-1, HCT116 and MGLVA1 cells. It may be possible to achieve more complete knock-down of gastrin in the esophageal cell-line by using a higher concentration of siRNA. However, previous data show that OE19 cells have gastrin levels above the upper range for esophageal tumors. Thus, the siRNAs are able to effectively knock-down gastrin expression even in cells expressing high levels of gastrin mRNA.

Example 8: Titration of siRNAs

In order to test the range of effectiveness of the siRNAs, the commercial tg8 siRNA (and scrambled control) were tested at a range of concentrations against HCT116 cells. The data are shown in Figure 6. Maximal knock-down was achieved with HCT116s using the highest concentration of siRNA (~40nM) and a dose-dependent response was seen. The full range

of *in vitro* synthesised siRNAs was also tested at a range of concentrations on Pan-1 cells in the experiment described above and shown in Figure 3.

Example 9: Time-course of gene expression

Pan1 cells were transfected with 40nM (undiluted) or 4nM (1:10) siRNAs. Gene expression was monitored at days 1, 4, 7 and 11. The data are shown in Figure 7. The experiment was repeated with HCT116 cells and gene expression measured at days 1, 3, 6 and 9. These data are shown in Figure 8.

The data show that the effects of the siRNAs are prolonged to at least day nine post-transfection. In Pan-1 cells, at the highest concentration of siRNA used, there is still 90% knock-down of the gastrin gene seven days after transfection and 80% knock-down at day eleven. (The apparently raised level at day four is an artifact due to one of the transfection replicates showing less effective knock-down for unknown reasons). At the lower concentration of siRNA, 85% knock-down of expression is maintained up to day seven, but drops to 53% by the eleventh day. knock-down of gastrin gene expression appears to fade more quickly in HCT116 cells than Pan-1 cells. This may be because HCT116 cells grow faster, or because HCT116 cells have higher levels of gastrin expression than Pan-1 cells. Even with HCT116 cells, there is still significant knock-down of gastrin gene expression as late as nine days post transfection.

Example 10: Downregulation of EGF-induced gene expression

Since EGF is known to up-regulate gastrin gene expression at the transcriptional level and contributes to growth of GI cancer cells we investigated the ability of gastrin siRNAs to inhibit this EGF-mediated gastrin expression. Figure 9 shows gastrin gene expression measured by real-time PCR in siRNA-transfected PAN-1 cells in the presence or absence of 10 μ g/ml EGF. The gastrin siRNA inhibited the induction of gastrin gene expression by EGF.

Example 11: Gastrin peptide expression

Pan-1 cells were harvested using trypsin/EDTA on day 3 following transfection with 40nM tg8 or scrtg8 siRNA. The transfection was carried out in duplicate for each siRNA. After washing, the cells were fixed using 4% formalin and stored at 4°C. The cells were used to make cytospins, permeabilised using Triton X100 and stained with a polyclonal rabbit antibody raised against amino acids 6-14 of progastrin. Binding of the primary antibody was detected using a fluorescent secondary antibody. Staining was detected using fluorescent microscopy.

Reduced levels of gastrin peptide expression were seen in the tg8-treated cells compared with the scrtg8-treated cells. However, there was still some residual staining of

the scrtg8 cells, compared with staining with a control irrelevant antibody suggesting that gastrin peptide knock-down lags behind gastrin mRNA knock-down. This is consistent with the biological effects of the siRNAs (see Examples 13 and 14).

Example 12: Inhibition of GFP-tagged gastrin protein expression by gastrin siRNA

The effect of siRNA transfection on gastrin protein expression was also analysed using HCT116 cells transfected with a GFP-tagged gastrin gene. The complete gastrin coding sequence was amplified by PCR and cloned upstream of the GFP coding sequence in the plasmid pHRGFP-C (Stratagene) under control of a CMV promoter using the BamH1 and XhoI restriction sites. A complete Kozak sequence was incorporated into the forward primer, involving modification of the 4th nucleotide of the gastrin coding sequence from C to G, indicated in the primer sequence by underlining. The primers used were as follows:

Forward: CGCGGATCCGCCGCCATGGGAGCGACTGTGTGTG (SEQ ID NO 10)

Reverse: CCGCCGCTCGAGGCCGAAGTCCATCCATC (SEQ ID NO 11)

The unmodified pHRGFP-C plasmid was used as a vector control. Since it lacks a Kozak sequence, no GFP expression was expected.

Dual transfection of plasmids and siRNAs was carried out by preparing the siRNA transfection mix as above but in half the volume, and pre-incubating 500ng of plasmid with 1 μ l Lipofectamine (InVitrogen) in 25 μ l serum-free medium. The two transfection reagents were then mixed immediately before addition to the cells. 24 hours following transfection, GFP expression was measured by flow cytometry.

4 replicates of each co-transfection were carried out. One replicate for each treatment is shown in Figure 11. The average percentage of fluorescent cells following transfection with pGasGFP and the control siRNA was 19.8 whilst only 4.8% of cells co-transfected with pGasGFP and the gastrin siRNA were positive. Thus, there was a significant reduction in the proportion of GFP-tagged gastrin-expressing cells following treatment with the gastrin siRNA ($p<0.0001$).

Example 13: Effects of gastrin siRNA on cell growth

In order to measure the effect of transfection with gastrin siRNAs on growth of cells in the presence or absence of EGF, MTT assays were carried out on cells transfected with the siRNAs. This assay is based upon the reduction of the colorless tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to the deeply colored red dye product in the mitochondria of metabolically active cells. During initial experiments, growth effects were not apparent immediately following transfection and day 5 was selected as the optimal time-point for the assay.

Pan1, HCT116, MGLVA1 or C170HM2 cells were transfected with 0.5 μ g of the tg8 or scrtg8 siRNAs, and treated with MTT for 4hrs on days 5 post-transfection. Six replicates

were performed for each siRNA and reduction of MTT was measured photometrically. Growth was measured on a minimum of 3 separate occasions for each cell-line and, whilst there was some variation between experiments, in all 4 cell-lines there was reduced growth in the tg8 compared with the scrtg8-treated cells. In the absence of EGF, the effects were most marked in the C170HM2 cells, reaching significance on each of the three occasions they were tested (% growth of 63.1, 75.0 and 68.5), whilst in the remaining three cell-lines, in the absence of EGF, significance was reached on only a proportion of occasions. However, in cells treated with EGF a significant difference in growth was seen in every independent experiment and the percentage reduction in growth was greater. Representative data for a single experiment for each cell-line is shown in Figure 11. Similar reductions in growth were seen in Pan1 and C170HM2 cells treated with two other gastrin siRNAs tg5 and tg7 (data not shown), suggesting that this effect is specifically related to down-regulation of the gastrin gene.

In addition, inhibition of growth was observed using a thymidine incorporation assay in Pan1 cells over a similar time-frame (data not shown).

Example 14: Apoptosis in gastrin siRNA-transfected cells

Since gastrin is known to be anti-apoptotic as well as acting as a growth factor, the effect of the siRNAs on apoptosis in the 4 cell-lines was also investigated. The degree of apoptosis in gastrin siRNA-treated cells was examined using an assay that involves treating cells with a fluorescent caspase 3-specific inhibitor (BioCarta). Caspase 3 is a key mediator in the initiation of the apoptotic cascade. The rationale behind this assay is that cells in which the caspase is activated are marked by binding of the fluorescent inhibitor, but prevented from continuing through the apoptotic pathway. Pan1, HCT116, MGLVA1 or C170HM2 cells were transfected with 0.5 μ g of the tg8 or scrtg8 siRNAs. On day 4 following transfection they were harvested using EDTA and treated with the FAM-DEVD-FMK reagent at the specified concentration. After 1 hour they were washed and fixed in 0.4% formalin, then analysed by flow cytometry. The percentage of positive cells was calculated relative to untreated cells. The data is shown in Figure 12. There was increased apoptosis in the cells treated with the gastrin siRNA compared with the control siRNA. In the absence of EGF the difference was significant in the Pan1 and C170HM2 cells. The effect was enhanced in the presence of EGF, with a significant difference seen additionally in the HCT116 cells. Significance was not reached in the MGLVA1 cells under either condition.

Example 15: Reduced PKB phosphorylation in cells transfected with the gastrin siRNA
The effect of the gastrin siRNA on phosphorylation of PKB/Akt was assessed in OE19 cells transiently transfected with tg8 or a scrtg8 siRNA at concentrations of 500ng/ μ l and

50ng/μl. Following transfection (72 hours), cells were assessed for PKB/Akt and phosphoryled PKB protein expression by Western Blotting. A significant decrease in levels of phospho-PKB/Akt compared to total PKB/Akt as determined via densitometry analysis of Western blots was seen (p=0.003) at the higher tg8 concentration (Figures 13a and b). Since phospho-PKB is an activator of growth and anti-apoptotic pathways in GI cancer cells (Lawlor and Alessi (2001) PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? *J. Cell Science* **114**:2903-2910), reduction in the levels of phospho-Akt in the cells may contribute to the reduced growth and increased apoptosis seen in gastrin siRNA-treated cells.

Example 16: Effect of gastrin siRNA on HB-EGF expression

H.pylori infection results in a pronounced hypergastrinaemia and upregulation of other human growth factors, including HB-EGF, that may contribute to the neoplastic progression associated with this infection (Parsonnet et al (1991) *Helicobacter pylori* infection and the risk of gastric carcinoma *N. Engl. J. Med.* **325**: 1127 -1131). Since HB-EGF expression is upregulated by gastrin, the effect of the gastrin siRNA on HB-EGF expression was investigated in three gastric cancer cell lines, MGLVA1, ST16 and AGS. Following transfection with the gastrin siRNA or control siRNA, HB-EGF expression was measured by real-time PCR. The results are shown in Figure 14. A significant decrease in HB-EGF gene expression was seen in all three cell-lines in the gastrin siRNA-treated cells compared with cells treated with the control siRNA.

Twenty-four hours after transfection with siRNAs, the cells were exposed to the cag⁺ vacA s1/m1 toxigenic *H. pylori* strain 60190 (ATCC no. 49503) for 24hrs. The results are shown in Figure 15. In the ST16 and AGS cells there was an increase in HB-EGF expression in response to *H. pylori* in control siRNA treated cells. In all three cell-lines, there was a significant reduction in HB-EGF expression in *H. pylori*-exposed cells treated with the gastrin siRNA compared with those treated with the control siRNA. This effect was also apparent at the protein level (see Figure 16). Exposure of cells to *H. pylori* (60190) results in upregulation of HB-EGF shedding which is inhibited in cells treated with the gastrin siRNA (target8).

Example 17: Effect of gastrin siRNA on XIAP expression

The X-linked inhibitor of apoptosis protein (XIAP) is a member of the inhibitor of apoptosis protein (IAP) family and a potent inhibitor of caspase-3, -6, -7 and 9. Since XIAP expression is raised in a number of cancers and is upregulated by phospho-Akt, the effect of the gastrin siRNA on XIAP expression in the gastric cancer cell line, AGS, was investigated. Following transfection with the gastrin (target 8) and control siRNA (scrambled tg8), XIAP expression was measured by real time PCR. The data are shown in Figure 17. Expression

was significantly reduced in the gastrin (target 8) siRNA-treated cells compared with the control siRNA (scrambled target 8) treated cells ($p<0.004$). Following transfection with the siRNAs, AGS cells were exposed to *H.pylori* (60910) for 24hrs and XIAP was again measured by real time PCR (see Figure 18). There was an increase in XIAP expression in control siRNA (scrambled target 8) treated cells exposed to 60190 and this was significantly reduced in gastrin siRNA-treated cells exposed to *H. pylori* ($p<0.005$). This inhibition of *H.pylori*-induced XIAP expression by the gastrin siRNA was also observed at the protein level (see Figure 19).

Example 18 27-mer gastrin siRNA

The 27-mer and tg8 gastrin siRNAs and the control siRNA (scrtg8) were used to transfect C170HM2 cells at a range of concentrations between 0.1nM and 40nM. Gene expression was monitored on day 1 by real time PCR. The data are shown in Figure 20. The 27-mer gastrin siRNA was as effective at knocking down gastrin gene expression as the tg8 siRNA.

Example 19: pSilencer cloning

Oligonucleotides were designed to encode tg5, tg7, tg8 and scrtg8 hairpin RNAs, with BamH1 and HindIII restriction sites to allow insertion into vector, pSilencer 2.1-U6 (Ambion Inc., Huntingdon, Cambridgeshire, UK). Their sequences are given in Table 2.

Target sequences and scrambled target sequences were assigned SEQ ID NOS are as follows: Tg5U is SEQ ID NO:12; Tg5L is SEQ ID NO:13; Tg7U is SEQ ID NO:14; Tg7L is SEQ ID NO:15; Tg8U is SEQ ID NO:16; Tg8L is SEQ ID NO:17; Scrtg8U is SEQ ID NO:18 and Scrtg8L is SEQ ID NO:19.

Table 2 Oligonucleotides used for cloning

Tg5	Upper	gatcccgaaggcaggaccatggctgtcaagagacagccatggccctgcttct ttttggaaa
	Lower	agctttccaaaaaagaaggcaggaccatggctgtcttgaacagccatggtc cctgcttcgg
Tg7	Upper	gatcccgaagaagaaggcctatggattcaagagaatccataggcttcttctt tttggaaa
	Lower	agctttccaaaaaagaagaaggcctatggattcttgaatccataggctt cttcttcgg
Tg8	Upper	gatcccgaagaaggcctatggatggattcaagagatccataggcttctt tttggaaa
	Lower	agctttccaaaaaagaagaaggcctatggatggatcttgaatccatagg cttcttcgg

Scrtg8	Upper	gatcccgcaagaaacgagggttcaagagaatacacccgtttctcgctt ttttggaaa
	Lower	agctttccaaaaaagcgaagaaacgagggttattcttcaaatacacccgtt tcttcgcgg

Each of the hairpin loops was cloned into the vector pSilencer 2.1 U6 obtained from Ambion (Huntingdon, Cambridgeshire, UK). An EcoRI/HindIII digest was used to visualize the insert. The structure of the gastrin siRNA stem-loop structure can be expressed from the recombinant pSilencer 2.1 U6 vector clone from Ambion.

The skilled artisan will immediately recognize the full scope encompassed by the present invention, which is exemplified and in no way limited by the above examples.

What is claimed is:

1. A gastrin gene-specific interfering ribonucleic acid (RNAi) molecule, comprising a ribonucleotide chain of up to about 90 nucleotides, the ribonucleotide chain comprising a targeting sequence of between about 19 and about 29 nucleotides of sufficient complementarity to a gastrin gene sequence to bind gastrin messenger RNA (mRNA).
2. The gastrin gene-specific interfering ribonucleic acid (RNAi) molecule according to claim 1 wherein targeting sequence is between about 19 and about 27.
3. The gastrin gene-specific interfering ribonucleic acid (RNAi) molecule according to claim 1 wherein targeting sequence is between about 19 and about 24.
4. The gastrin gene-specific interfering ribonucleic acid (RNAi) molecule according to claim 1 wherein targeting sequence is between about 19 and about 21.
5. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 4, wherein the ribonucleic acid (RNAi) molecule inhibits gastrin gene expression.
6. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 5, wherein the inhibition of expression comprises inhibition of translation of the gastrin gene.
7. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 6, wherein the inhibition of expression comprises degradation of gastrin mRNA.
8. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 7, comprising a second nucleotide sequence of between about 19 and about 29 nucleotides having sufficient complementarity to bind the targeting nucleotide sequence.
9. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 7, wherein the second nucleotide sequence is between about 19 and about 27.
10. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 7, wherein the second nucleotide sequence is between about 19 and about 24.
11. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 7, wherein the second nucleotide sequence is between about 19 and about 21.
12. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 11, comprising a modified nucleotide.
13. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 12, comprising an internucleotide linkage which is not a 3'-5' phosphate linkage.
14. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 13, wherein the second nucleotide sequence is covalently joined to the ribonucleotide chain via a linker molecule.

15. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 14, wherein the sequence complementarity to a gastrin coding sequence comprises at least seventeen consecutive nucleotides of a nucleotide sequence selected from the group consisting of: AAGCTTCTTCCAAGCCCCGCT (SEQ ID NO:1); AAGAAGCAGGGACCATGGCTG (SEQ ID NO:2); AAGAAGAAGAAGCCTATGGAT (SEQ ID NO:3); AAGAAGAAGCCTATGGATGGA (SEQ ID NO:4); AAGAAGCCTATGGATGGATGG (SEQ ID NO:5); AAGCCTATGGATGGATGGACT (SEQ ID NO:6); AAGAGATGTAAGGCCAGGCCG (SEQ ID NO:7); AAGCGAAGAACGAGGTGTAT (SEQ ID NO:8); and AAGAAGAAGCCTATGGATGGACT (SEQ ID NO:9).
16. The gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 15, comprising a nucleotide sequence selected from the group consisting of the sequences listed in Table 2 (SEQ ID NOS:12-19).
17. A pharmaceutical composition comprising the gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 16, and a physiologically acceptable carrier.
18. A pharmaceutical composition comprising the gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 17, and a peptide covalently linked thereto.
19. A method of treating a patient suffering from a gastrin-promoted disease or condition, comprising administering to the patient an effective amount of a gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 18.
20. The method according to claim 19, wherein the gastrin-mediated disease or condition is selected from the group consisting of a gastrin-promoted tumor, gastric-esophageal reflux disease (GERD), Barrett's esophagus, , atrophic gastritis, gastric ulceration, duodenal ulceration, Zollinger-Ellison syndrome, hypergastrinemia, pernicious anemia, pre-malignant conditions like Barrett's esophagus and *H. pylori* infection.
21. The method according to any one of claims 19 to 20, wherein the gastrin-promoted tumor is a tumor selected from the group consisting of a colonic adenoma, a pan-intraepithelial neoplasia, a gastric neoplasia, an intestinal tumor, a pancreatic tumor, a medullary thyroid carcinoma, an hepatic tumor, a pulmonary tumor, an ovarian tumor, tumors of brain origin and a tumor of neuroendocrine origin.
22. A method of reducing gastrin gene expression in a cell, comprising contacting the cell with a gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 16.
23. A nucleic acid vector expressing a gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 16.

24. A host cell carrying a nucleic acid vector expressing a gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 16.
25. A pharmaceutical composition comprising a nucleic acid vector expressing a gastrin gene-specific interfering ribonucleic acid molecule (RNAi) according to any one of claims 1 to 16, and a physiologically acceptable carrier.

FIG. 1

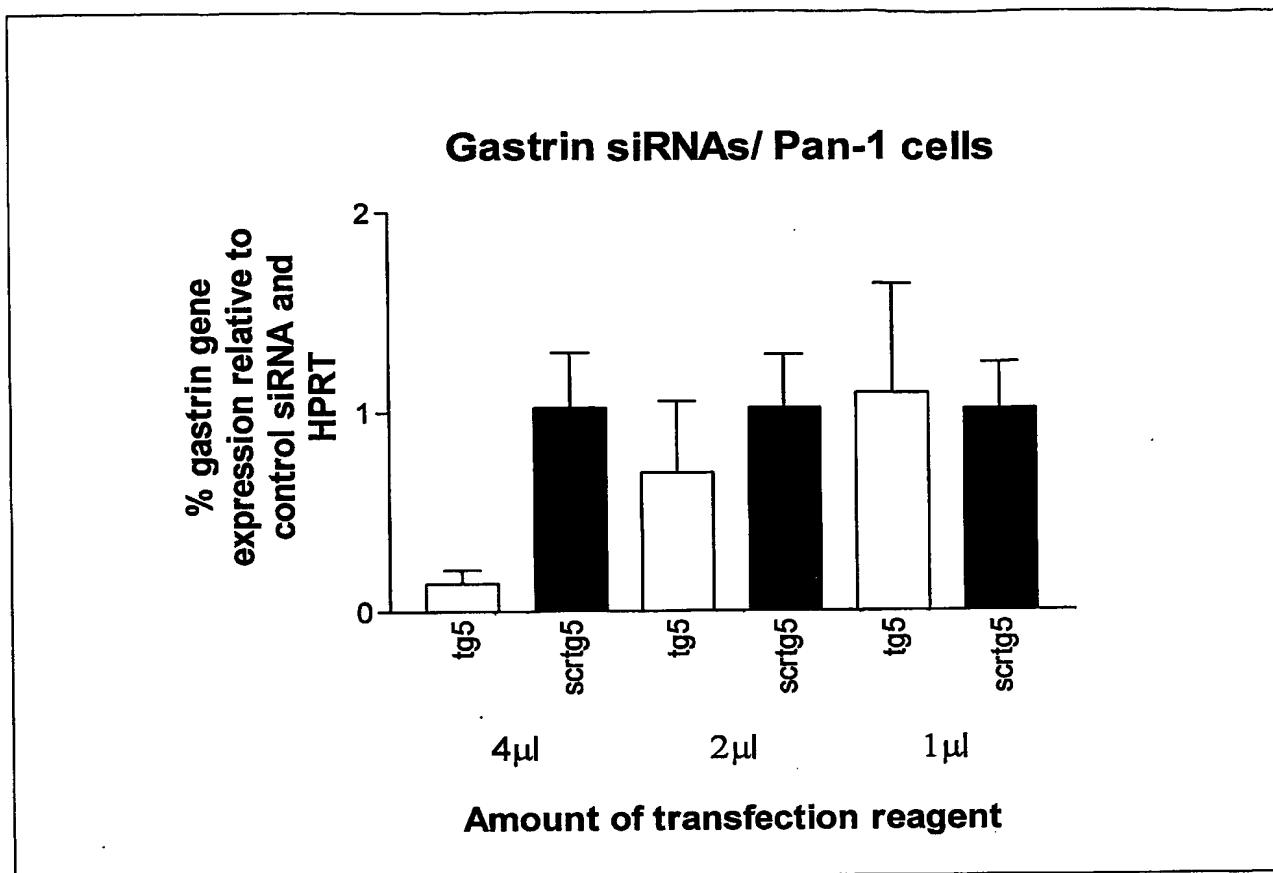


FIG. 2

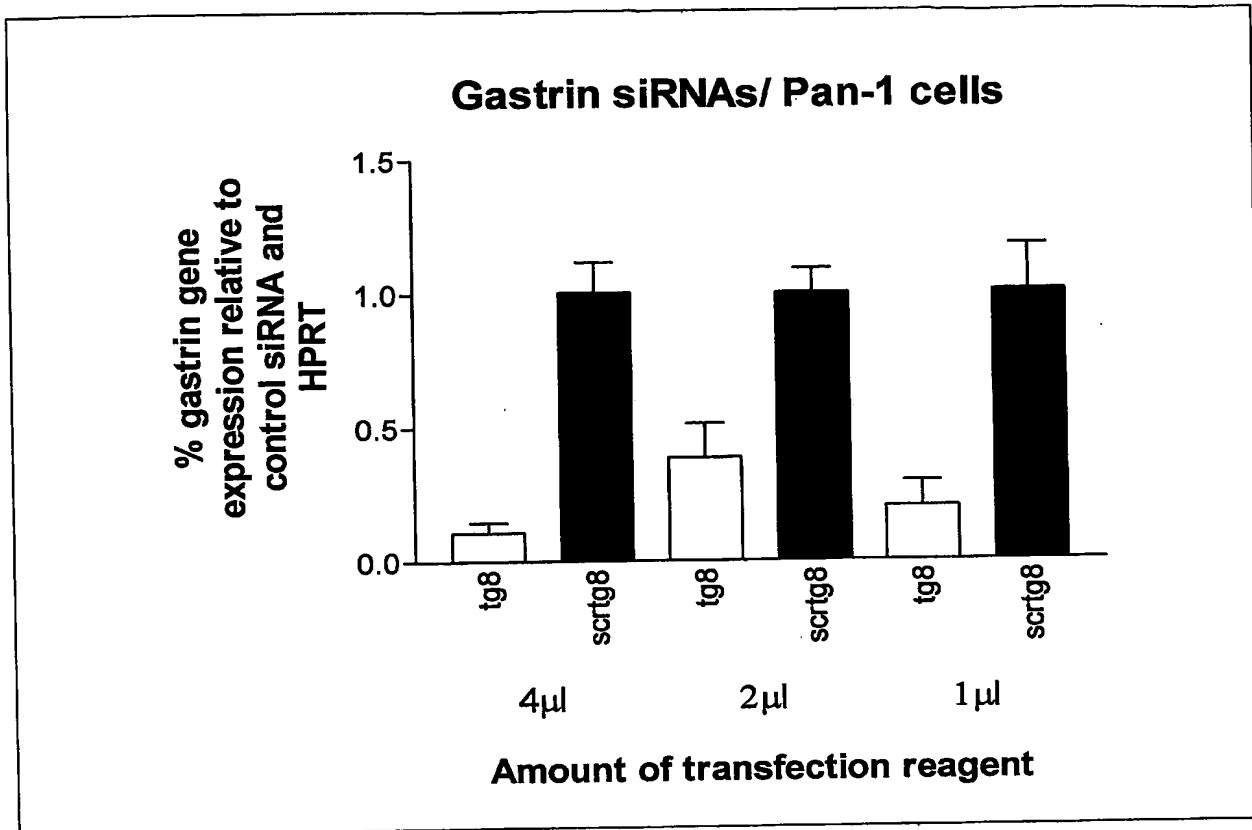


FIG. 3

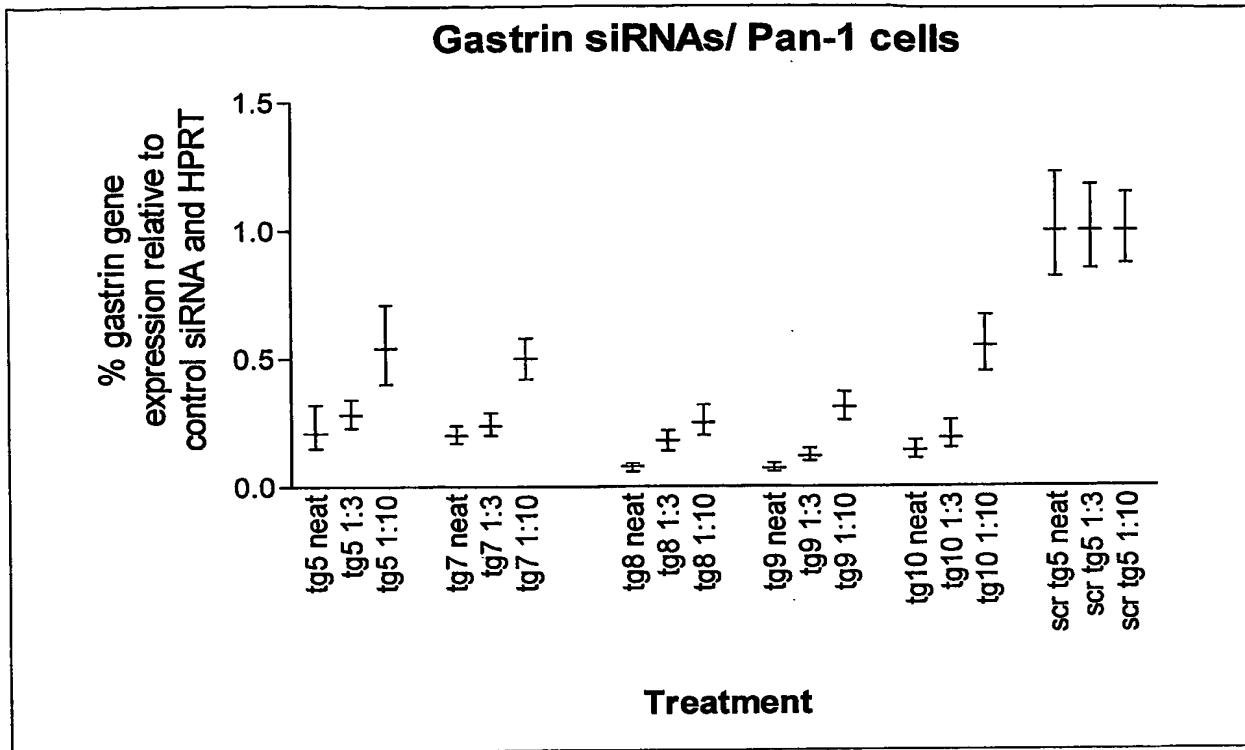


FIG. 4

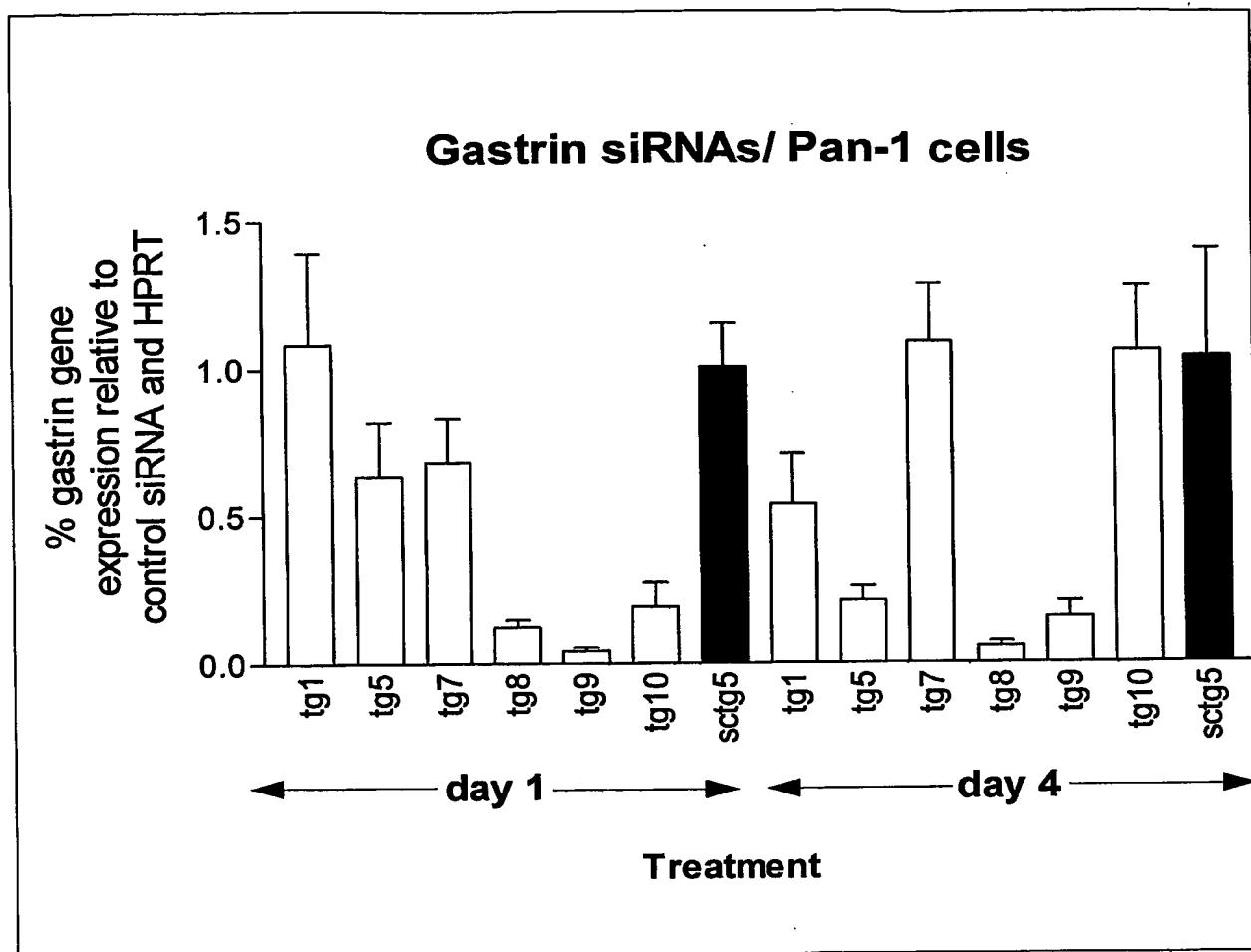


FIG. 5

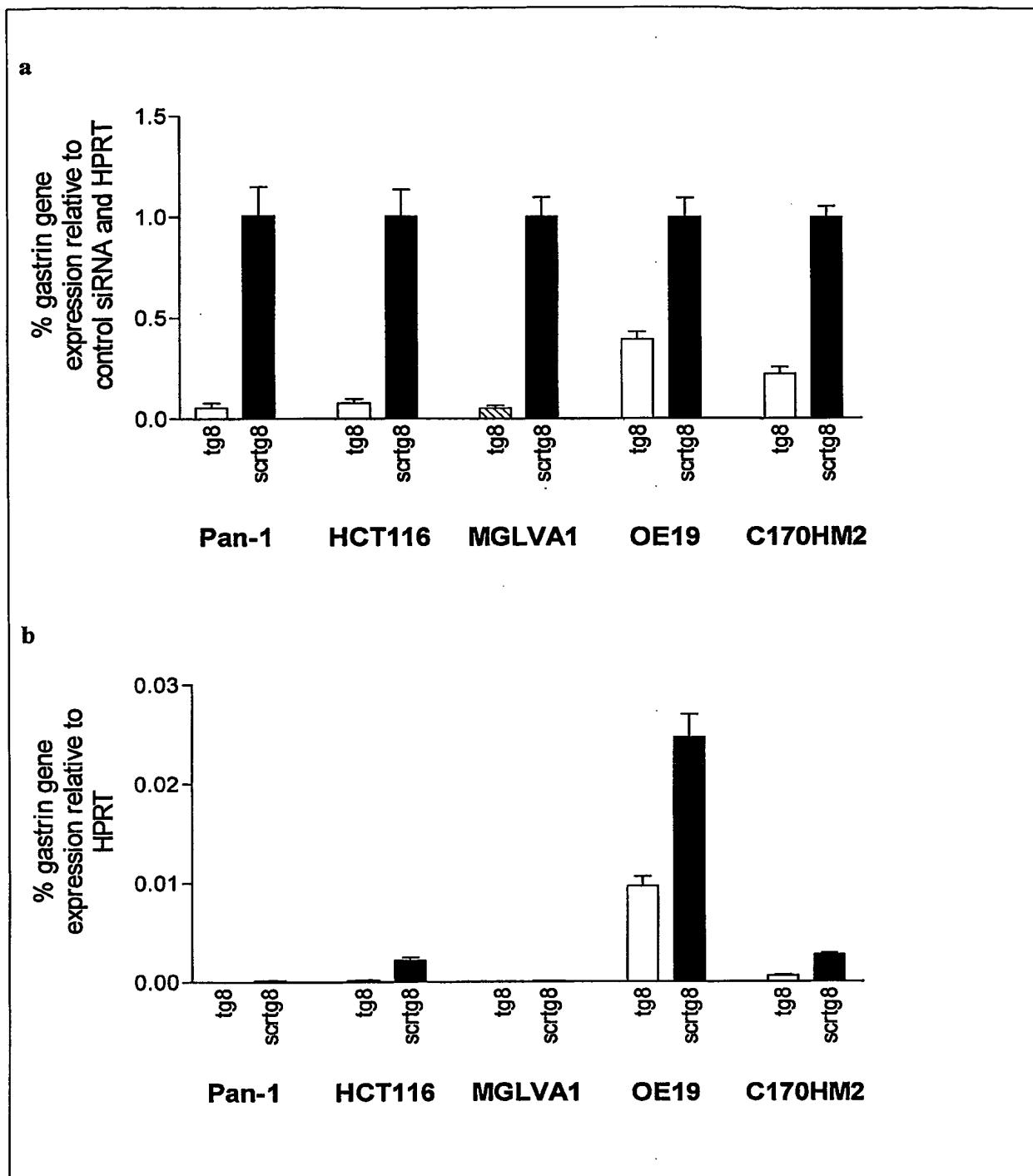


FIG. 6

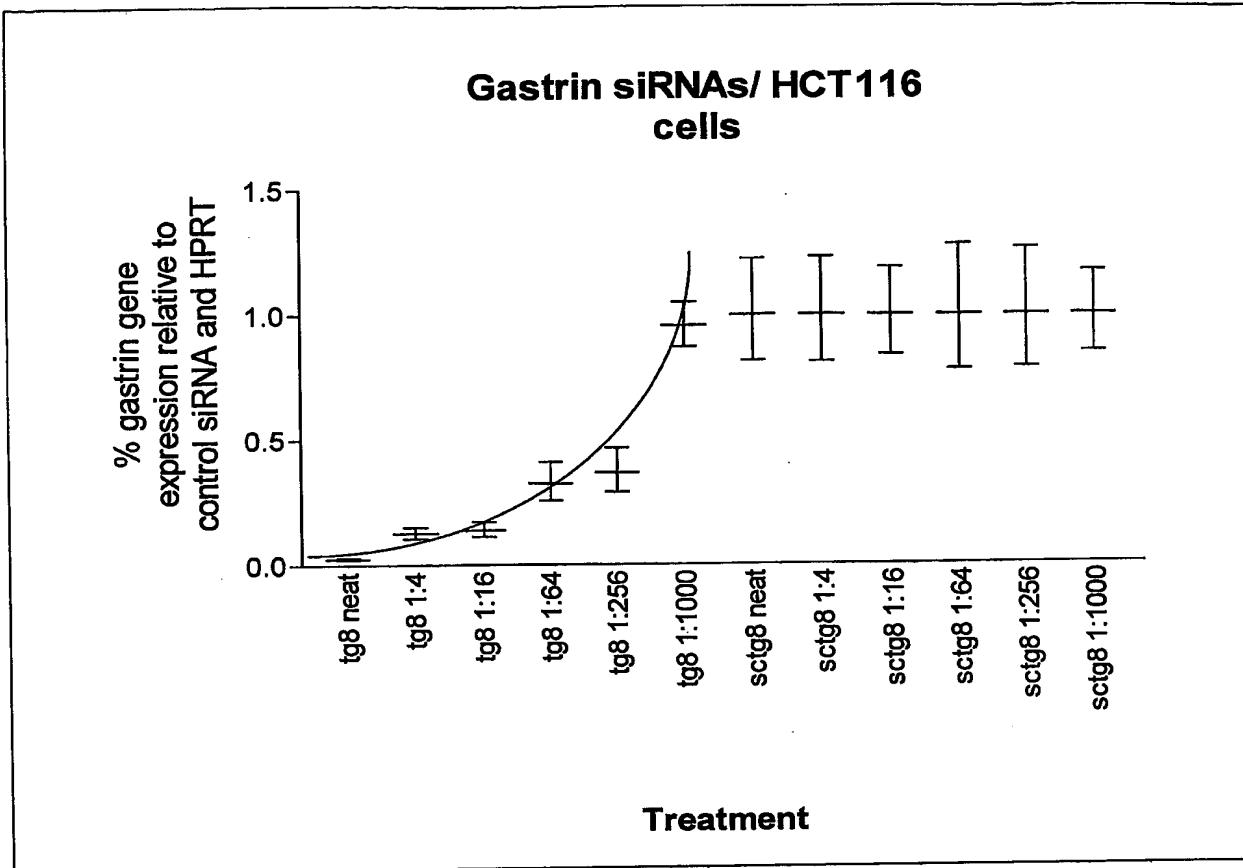


FIG. 7

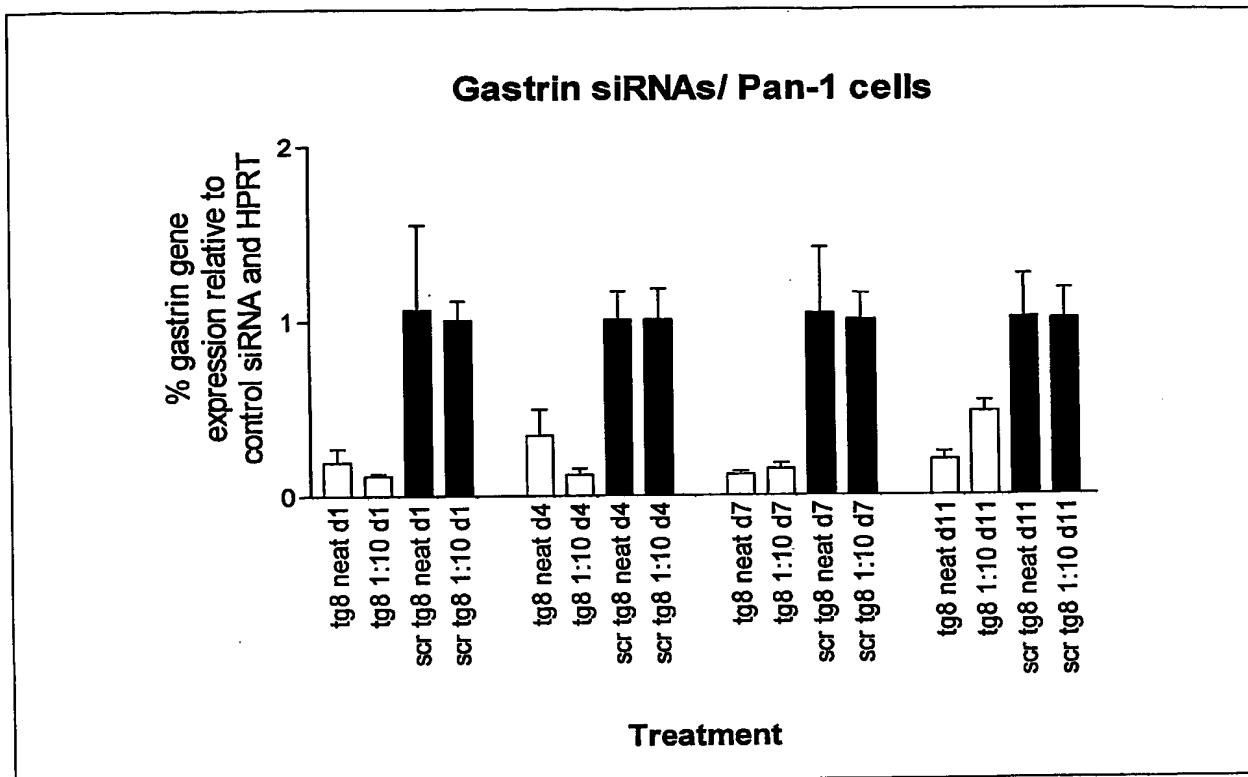


FIG. 8

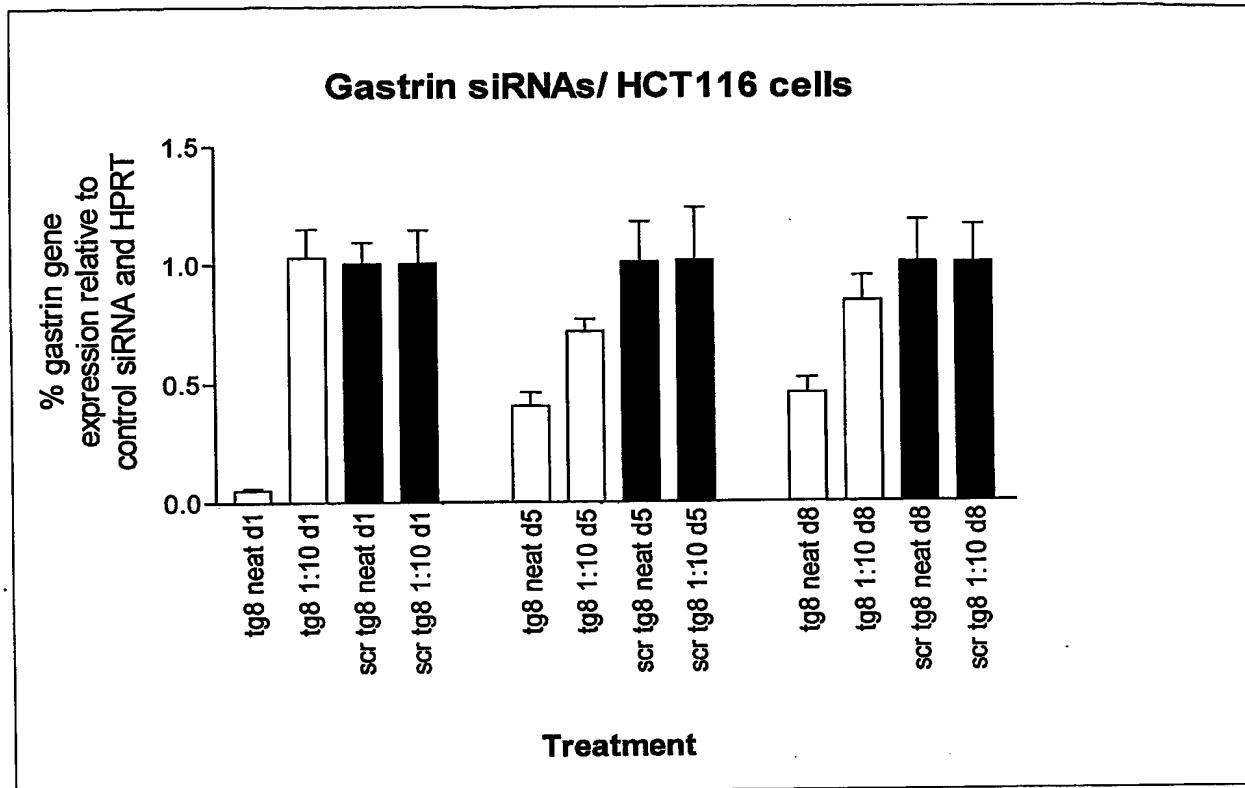


FIG. 9

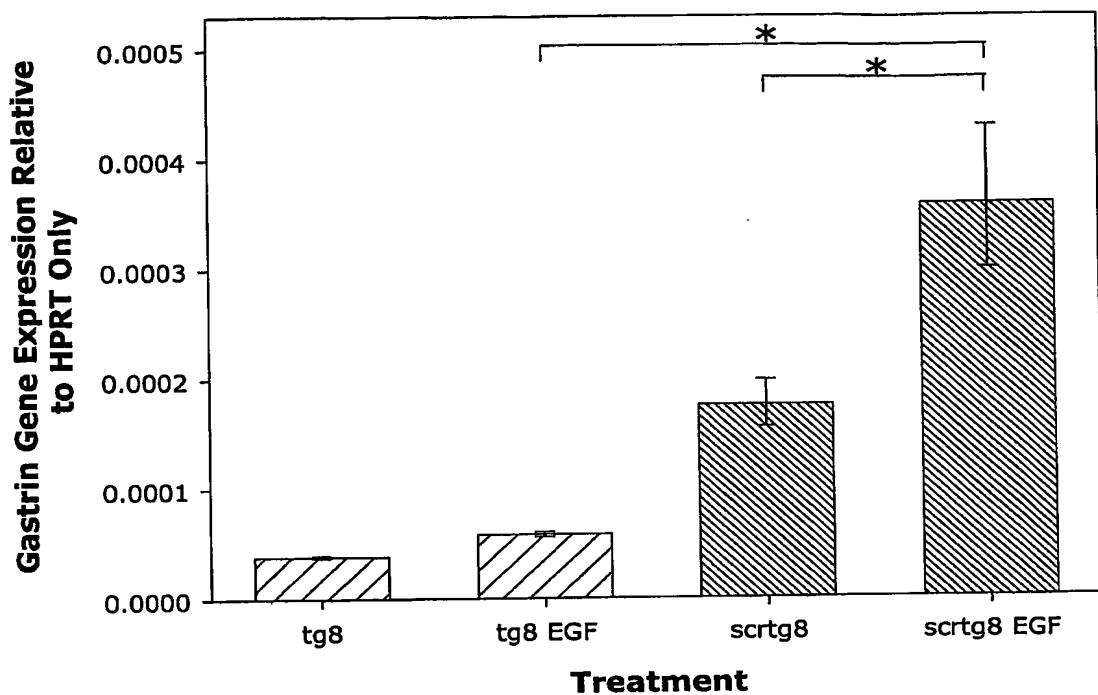
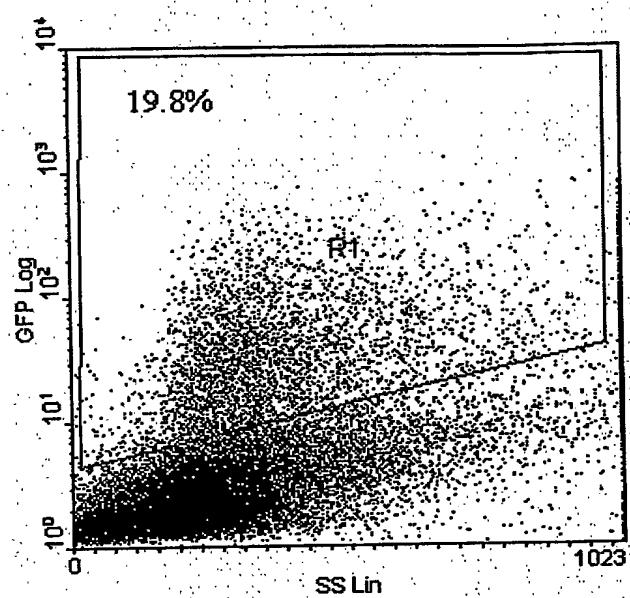


FIG. 10

a



b

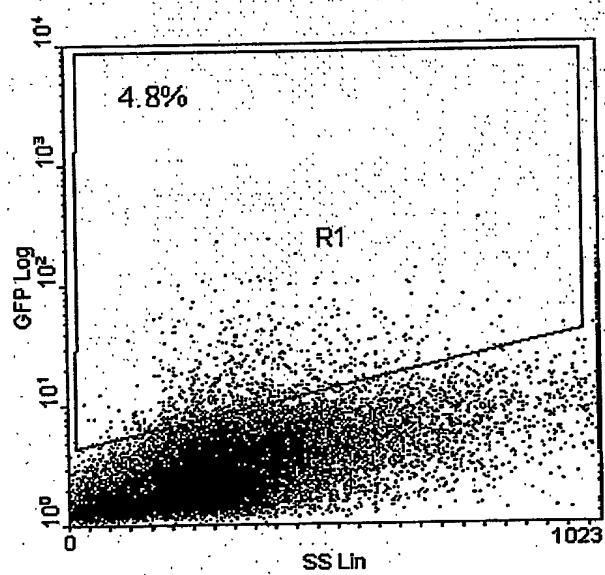
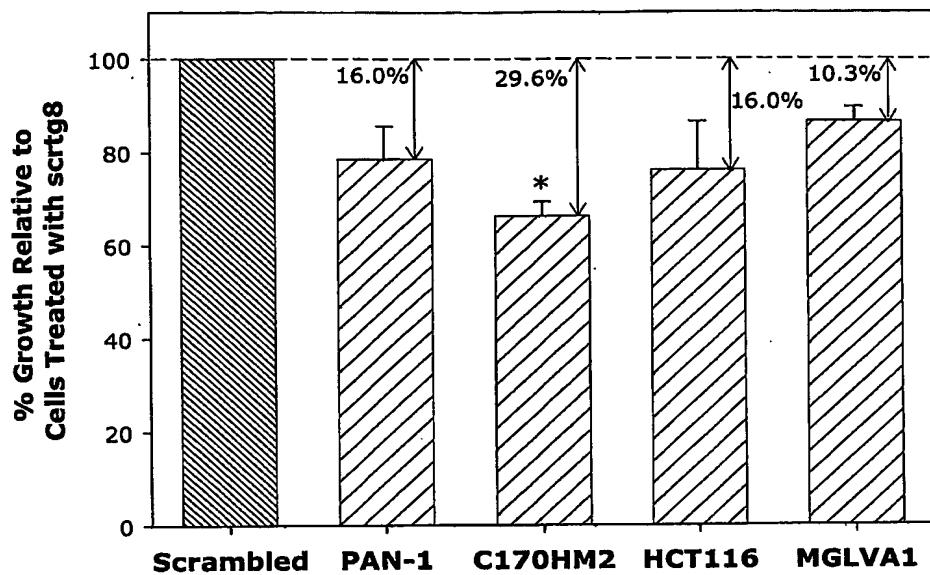


FIG. 11

a



b

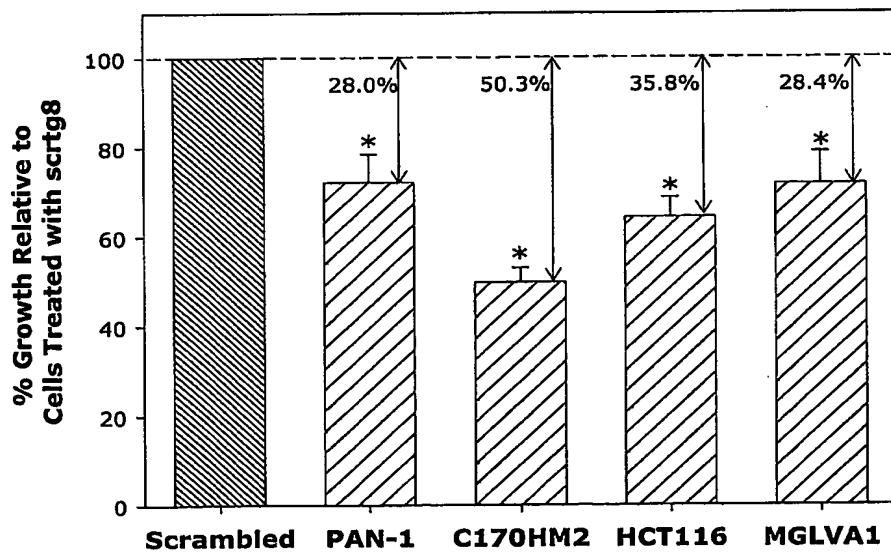
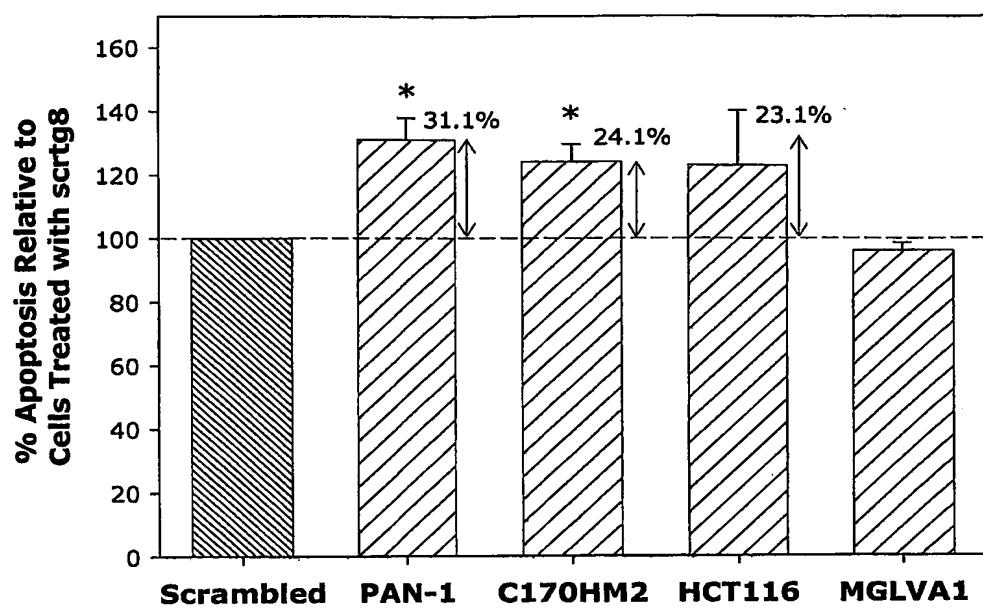


FIG. 12

a



b

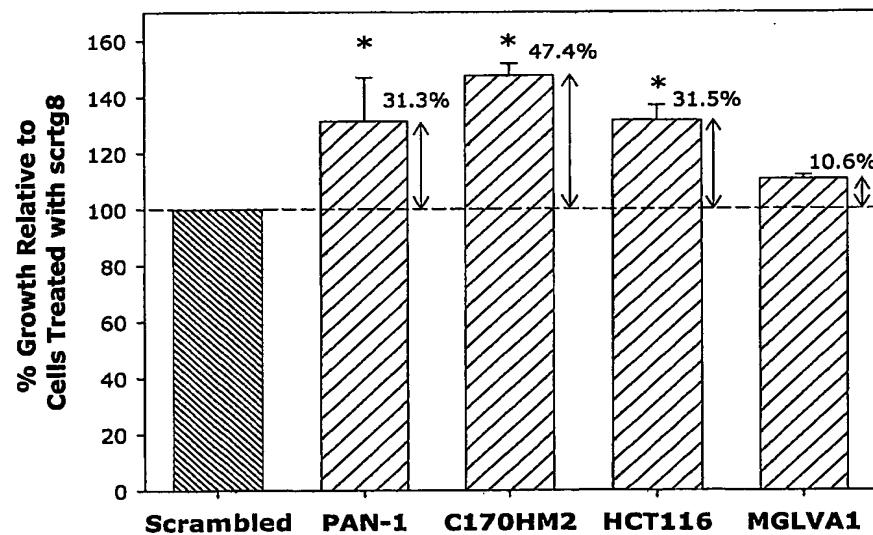
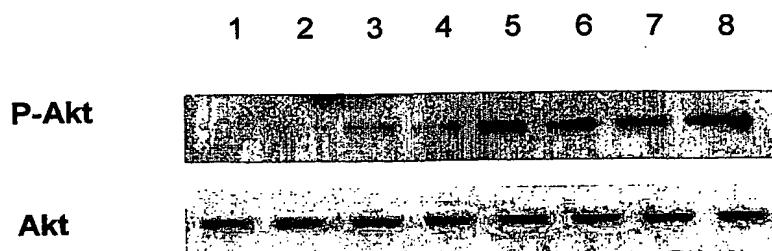


FIG. 13

A



B

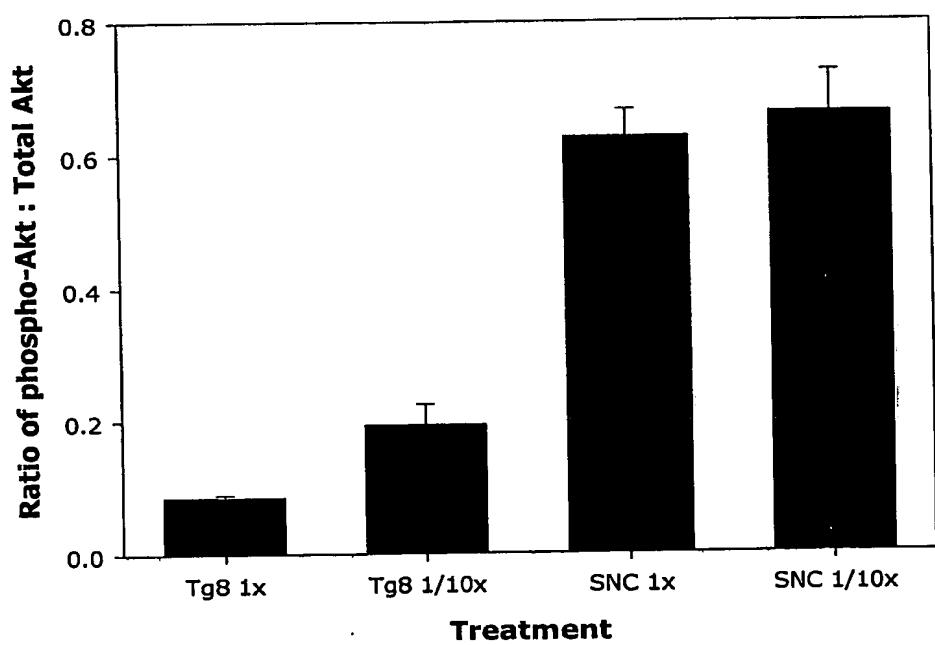


FIG. 14

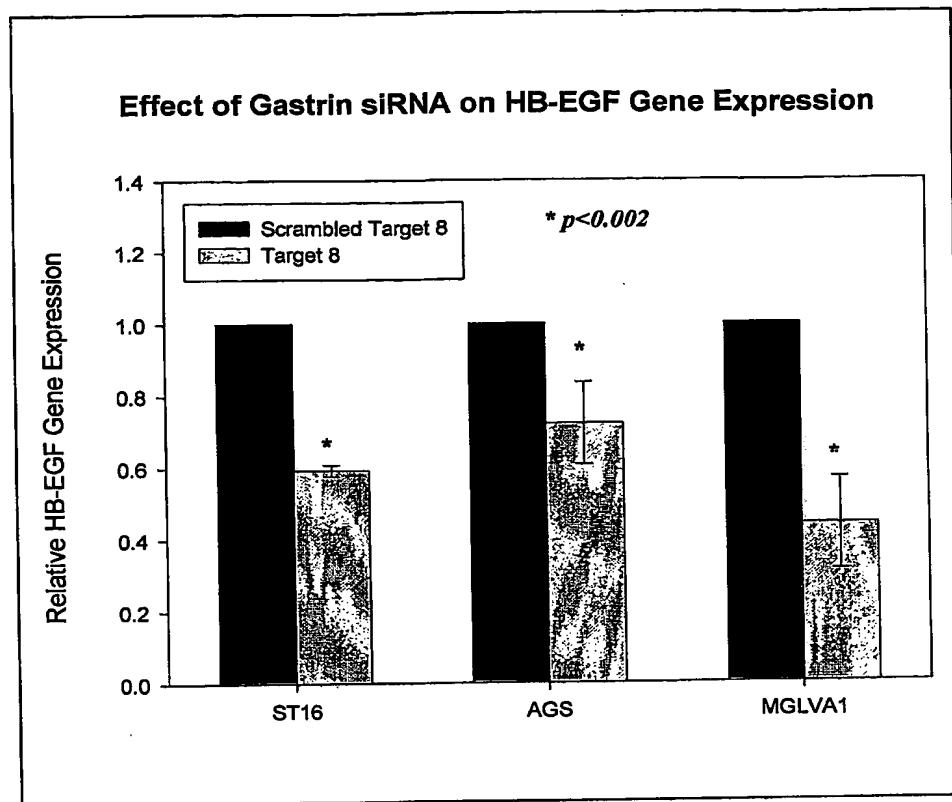


FIG. 15

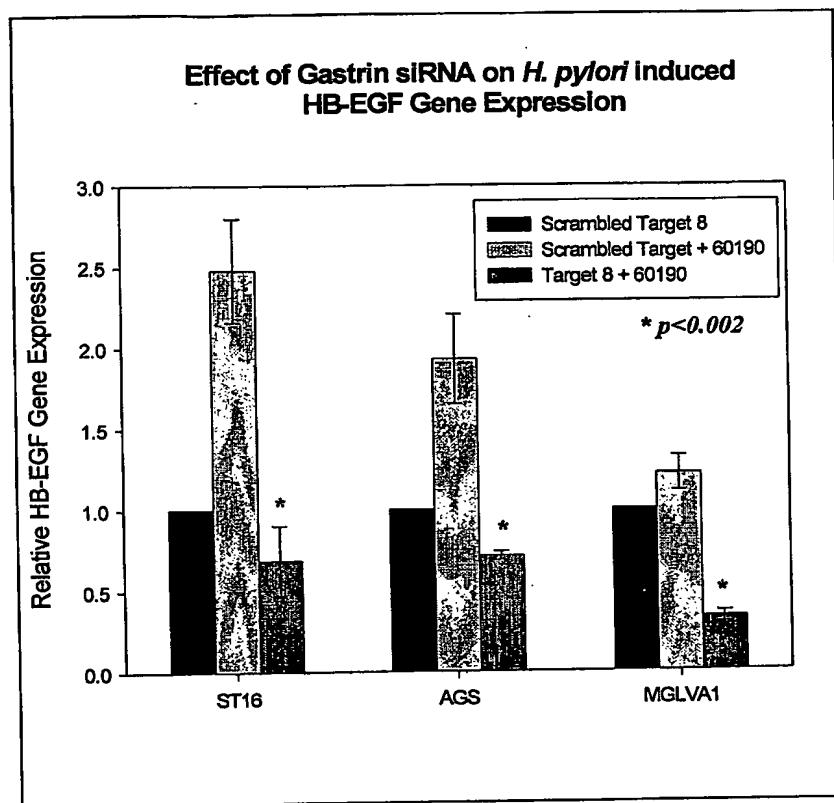


FIG. 16

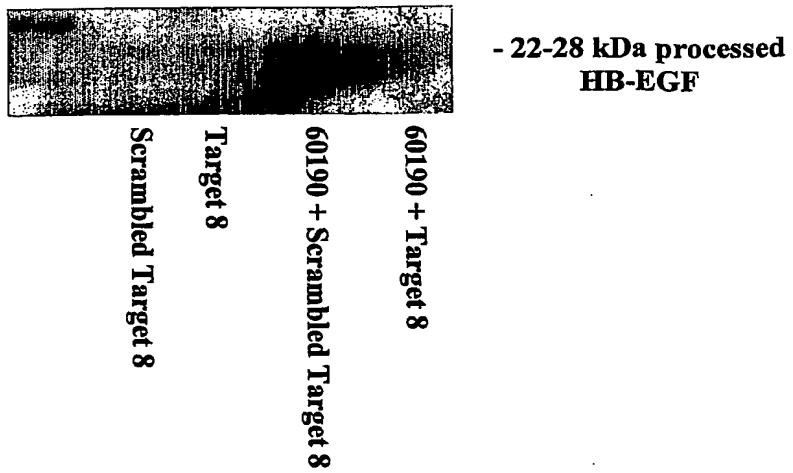


FIG. 17

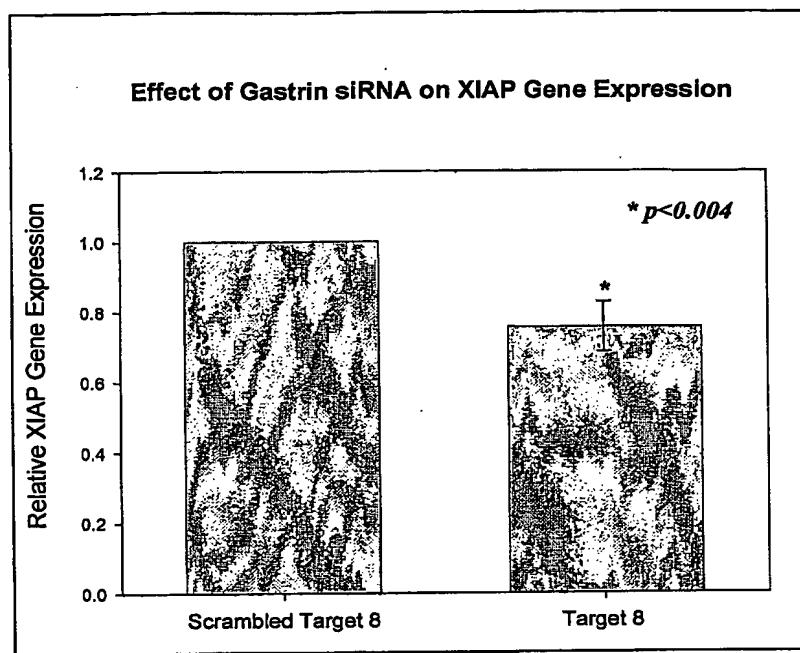


FIG. 18

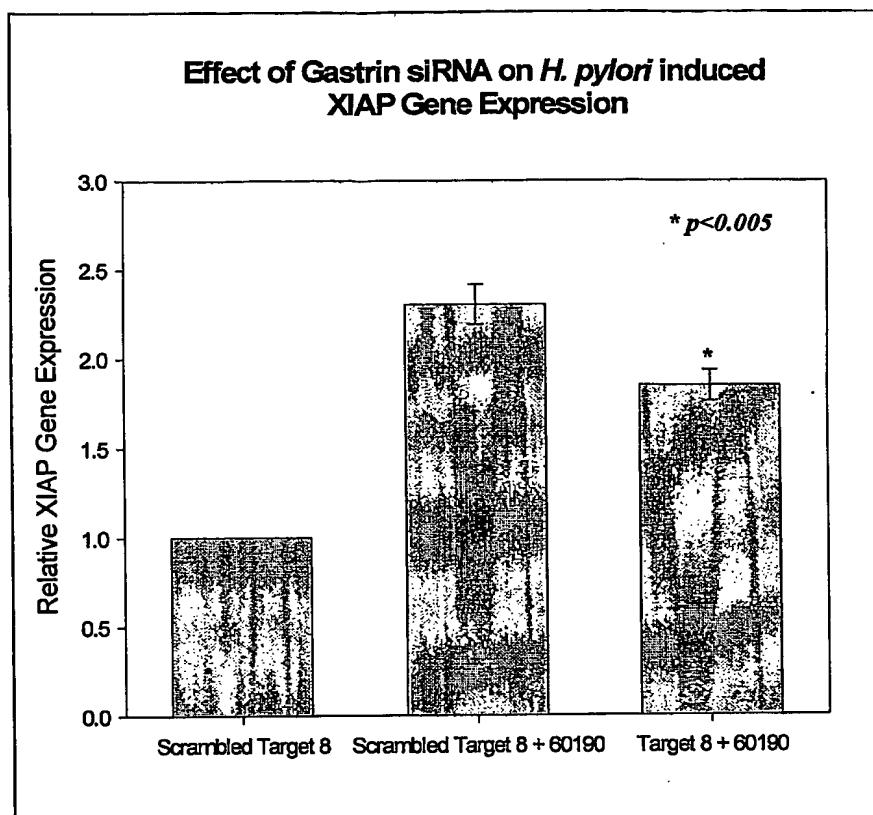


FIG. 19

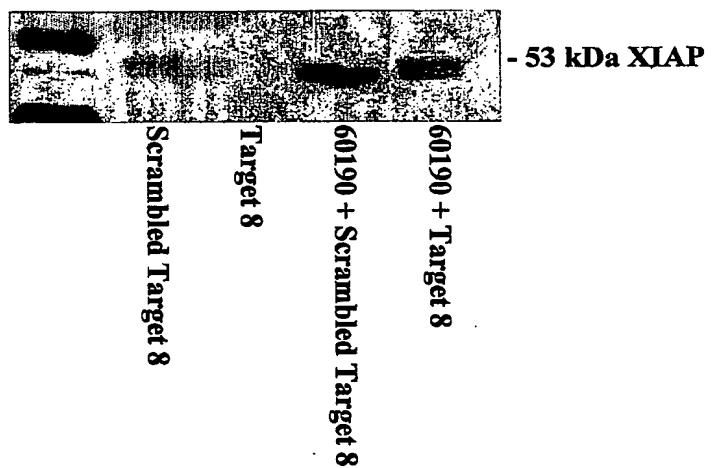


FIG. 20

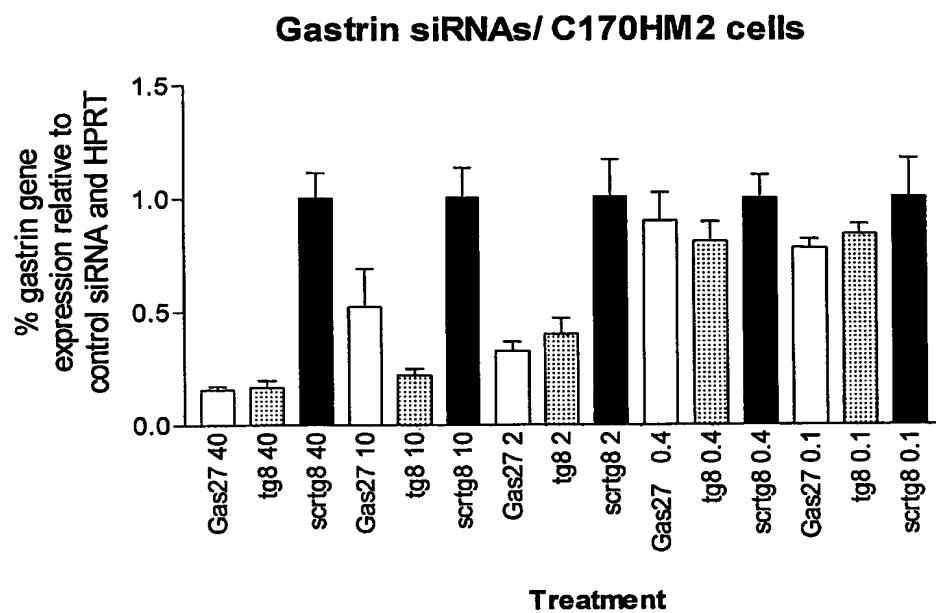
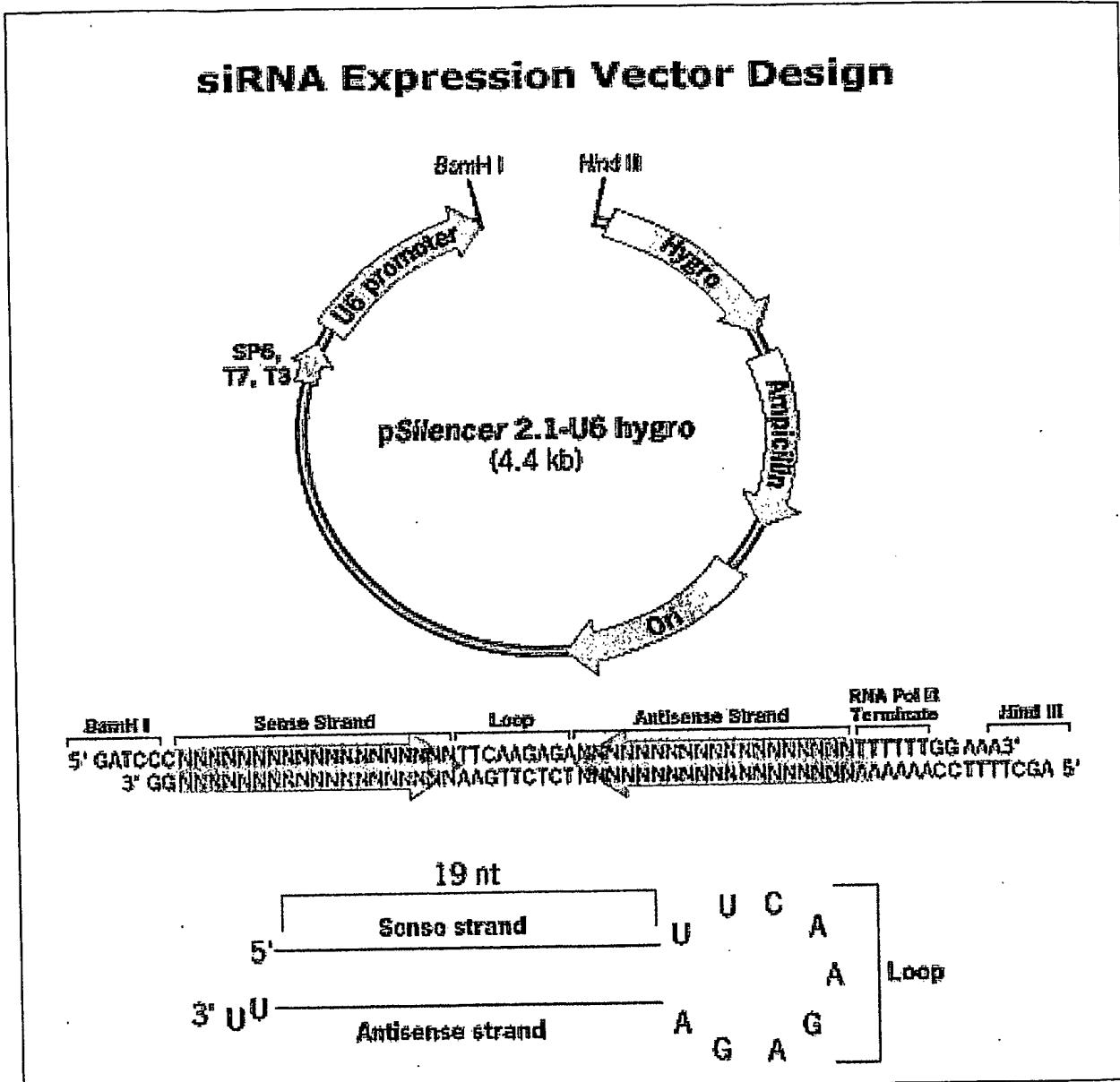


FIG. 21



Aphoton 0072.WorkFile.txt

Organization Applicant

Street : 8 Penn Center, Suite 2300; 1628 JFK Boulevard
City : Philadelphia
State :
Country : Philadelphia
PostalCode : PA 19103
PhoneNumber :
FaxNumber :
EmailAddress :
<110> OrganizationName : Aphoton Corp.

Application Project

<120> Title :
<130> AppFileReference :
<140> CurrentAppNumber :
<141> CurrentFilingDate :

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21

Sequence

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Sequence

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Sequence

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Aphoton 0072.workFile.txt

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Sequence

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SequenceDescription :

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Seite 2

60

Aphoton 0072.WorkFile.txt

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Sequence
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Aphoton 0072.WorkFile.txt

SequenceDescription :

Sequence

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gaaa 64
<212> Type : DNA
<211> Length : 64
SequenceName : oligonucleotide for cloning Tg5 upper SEQ ID No 12
SequenceDescription :

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(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

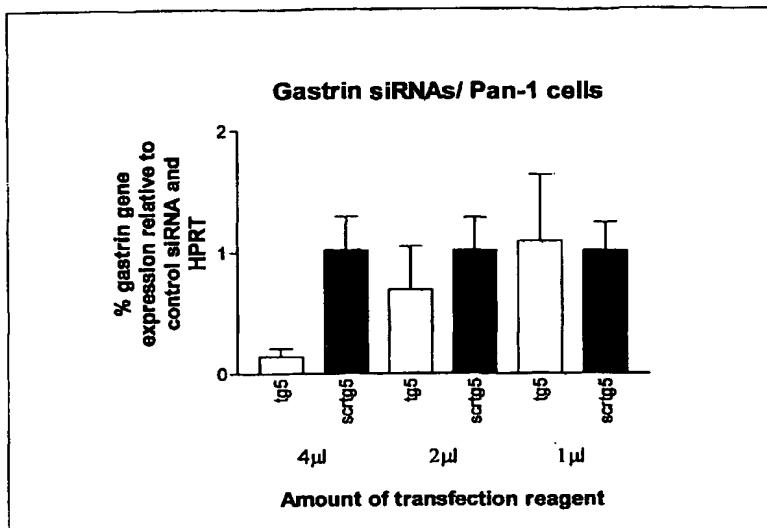
Declaration under Rule 4.17:
— of inventorship (Rule 4.17(iv))

Published:
— with international search report

[Continued on next page]

(54) Title: GASTRIN-SPECIFIC INTERFERING RNA

WO 2006/016275 A3



(57) Abstract: The invention provides gastrin-specific interfering ribonucleic acid (RNAi) molecules that down-regulate the gastrin hormone gene. The RNAi molecules of the invention are double-stranded RNA molecules that can include modified bases and non-phosphate linkages between the bases. The complementary strands of the RNAi molecules of the invention can be linked by a nucleotide chain or by non-nucleotide linkers. The RNAi molecules of the invention can be formulated in pharmaceutical compositions useful in a method for treating gastrin-mediated diseases or conditions including gastrin-mediated tumors, GERD, Zollinger-Ellison syndrome, hypergastrinemia, pernicious anemia, gastric ulceration, duodenal ulceration, and *H. pylori* infection.



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International application No

IB2005/002686

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHEDMinimum documentation searched (classification system followed by classification symbols)
 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	<p>SMITH J P ET AL: "Antisense oligonucleotides to gastrin inhibit growth of human pancreatic cancer" CANCER LETTERS, NEW YORK, NY, US, vol. 135, no. 1, 8 January 1999 (1999-01-08), pages 107-112, XP002357825 ISSN: 0304-3835 abstract page 107, right-hand column, paragraph 2 page 109, right-hand column, paragraphs 2,3</p> <p>-----</p> <p style="text-align: center;">-/-</p>	1-25

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Date of the actual completion of the International search

16 February 2006

Date of mailing of the international search report

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Authorized officer

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National application No
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Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SINGH P ET AL: "TUMORIGENICITY OF A HUMAN COLON CANCER CELL LINE IS SIGNIFICANCE REDUCED IN VIVO, IN THE PRESENCE OF COLON CANCER CELLS OVEREXPRESSING ANTISENSE GASTRIN mRNA: ROLE OF INHIBITORY (ENDOCRINE) FACTORS IN THE OBSERVED BYSTANDER EFFECTS" GASTROENTEROLOGY, ELSEVIER, PHILADELPHIA, PA, US, vol. 114, no. 4, PART 2, 15 April 1998 (1998-04-15), page A680, XP008056925 ISSN: 0016-5085 abstract	1-25
Y	WATSON S ET AL: "GASTRIN INHIBITION INCREASES THE POTENCY OF CYTOTOXIC AGENTS IN PANCREATIC CANCER" GASTROENTEROLOGY, ELSEVIER, PHILADELPHIA, PA, US, vol. 122, no. 4, SUPPL 1, April 2002 (2002-04), page A241, XP008056958 ISSN: 0016-5085 abstract	1-25
Y	WO 03/010180 A (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 6 February 2003 (2003-02-06) the whole document	1-25
Y	WO 2004/042061 A (STREBHARDT, KLAUS; SPAENKUCH-SCHMITT, BIRGIT) 21 May 2004 (2004-05-21) the whole document	1-25
P, X	GRABOWSKA A ET AL: "Downregulation of the gastrin gene using small interfering RNA" REGULATORY PEPTIDES, vol. 122, no. 1, September 2004 (2004-09), page 46, XP009061814 & 15TH INTERNATIONAL SYMPOSIUM ON REGULATORY PEPTIDES; TOULOUSE, FRANCE; SEPTEMBER 19 -22, 2004 ISSN: 0167-0115 abstract	1-25
	-/-	

INTERNATIONAL SEARCH REPORT

International application No
IB2005/002686

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	<p>TAKHAR A S ET AL: "The role of gastrin in colorectal carcinogenesis." THE SURGEON : JOURNAL OF THE ROYAL COLLEGES OF SURGEONS OF EDINBURGH AND IRELAND. OCT 2004, vol. 2, no. 5, October 2004 (2004-10), pages 251-257, XP009061845 ISSN: 1479-666X abstract page 252, right-hand column, paragraph 2 page 255, left-hand column, last paragraph - right-hand column, paragraph 5 -----</p>	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB2005/002686

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: 19-22 because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 19 to 22 are either directed to or embrace a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT**Information on patent family members**

International application No

'IB2005/002686

Patent document cited in search report	Publication date		Patent family member(s)	Publication date
WO 03010180	A 06-02-2003	CA EP JP	2454183 A1 1409506 A1 2005508306 T	06-02-2003 21-04-2004 31-03-2005
WO 2004042061	A 21-05-2004	AU	2003283374 A1	07-06-2004